



# AN IN VITRO MODEL TO STUDY HUMAN NEUROMUSCULAR JUNCTION DEVELOPMENT

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MEDICINE IN INDUSTRIAL SPECIALIZATION BIOMEDICINE Aalborg University, 2019

# TITLE PAGE

Title	An <i>in vitro</i> model to study human neuromuscular junction development
Project group	9028
Semester	9 <sup>th</sup> and 10 <sup>th</sup> semester – Medicine in Industrial Specialization, Biomedicine, Aalborg University
Project period	September 2018 – May 2019
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External supervisor	Abigail Mackey
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# ABSTRACT

Sarcopenia is medical condition characterised as loss of muscle mass, strength or function. It is elevating problem between elderly as age is one of the risk factors. Sarcopenia is caused by multiple factors varying from increase in oxidative stress, low level inflammation, poor dietary habits, lack of physical activity to degeneration in neuromuscular junction (NMJ). NMJs are responsible for transduction of signal from presynaptic motor neuron (MN) to postsynaptic myofiber. Currently there is no specific treatment for NMJ degeneration and development of human in vitro model is crucial step for understanding cellular as well as molecular processes. In this study, effect of agrin and glial derived neurotrophic factor (GDNF) on differentiation and acetylcholine receptors (ACHRs) production of primary myoblasts were tested. Moreover, heterogenous co-culture of human myoblasts and rat neurons has been established. Agrin as a molecule produced mostly by neurons had no positive effect on myoblasts' ACHR clustering and differentiation according to immunocytochemistry and qPCR. However, results from ICC displayed that when GDNF were added to the culture myoblast created slightly larger myoblast with multiple nuclei. qPCR supported the results with upregulation of ACHR genes such as CHRND, CHRNG and MUSK. Co-culture of human myoblast and rats' neurons has been also established. However, there was no obvious evidence of matured ACHRs in neither of our experiments and therefore it is a question of future research.

## SARCOPENIA

Sarcopenia is defined as the loss of muscle mass and function that occurs with aging, which is aggravated by factors such as reduced physical activity, obesity and insulin resistance. It has been recently estimated that the prevalence of sarcopenia is approximately 10 % of the total world population to 33 % in elderly community (Marty, Liu, Samuel, Or, & Lane, 2017; Shafiee et al., 2017). Such individuals are at a high risk of adverse health outcomes, including reduced mobility, functional disability and metabolic disorders, which all have profound consequences to the national healthcare systems. The pathophysiology is multifactorial, beside many elements just as low caloric intake, hormonal decline, myostatin enhancement and intracellular oxidative stress, one of the factor is muscle fibre denervation. Mostly affected are motor neurons which innervates type II fast twitch muscles what requires the recruitment of the remaining motor units to enlarge, branch out and increase the burden of work. Such a process leads to transformation of fibres from type II to type I (Marty et al., 2017). Fortunately, denervation – innervation cycle results to only slight decrease in strength and control in young organism. However, this compensation mechanism starts to fail with aging. The reason behind the progressive denervation with aging is unknown, but there are indications pointing at the aging processes located in synaptic connection between muscle and the end of neuron branch called neuromuscular junction (Gonzalez-Freire, de Cabo, Studenski, & Ferrucci, 2014).

# NEUROMUSCULAR JUNCTIONS

## DEVELOPMENT OF NMJS

NMJ development is still a research objective for its unclear mechanism because of the lack of human studies. Lasting dispute between the scientists lays in the question whether the neurons or muscle cells determine where and how NMJs are formed. The most accepted theory in mammals so far is called prepatternig, where acetylcholine receptors (ACHRs) located on postsynaptic domain appears and are equally distributed in central region of the muscle forming a band around the fibre before innervation (fig.1). Subsequently, the prepatterned domain is recognized by nerve terminals, which overlap some of the clusters on the surface of the muscle fibre and are enlarged and stabilized while primitive clusters of ACHR in non-innervated parts disappear (Legay & Mei, 2017; Wu, Xiong, & Mei, 2010).



Figure 1: Pre-patterning theory of creation of neuromuscular junction. ACHRs are evenly distributed across the myotube and clustered at the location of connecting motor neuron. Agrin secreted by MNs induced agrin/LRP4/Musk pathway.

In the prepatterning theory, molecular signalling is therefore initiated by muscle cells and progressively maintained by motor neurons and Schwann cells. Several signalling molecules are organized in both anterograde and retrograde manner, but only few of them play substantial role.

From molecular point of view, the best characterised pathway is agrin/Lrp4/Musk pathway (Wu, Xiong, & Mei, 2010). The glycoprotein agrin is secreted by all 3 types of the cells present at NMJ (motor neurons, Schwann cells and myoblasts). However, neural isoform is about 1000 times more effective in its function of ACHR clustering. Neural agrin is synthetized in cell bodies of motor neurons and transferred along the axons, secreted to the intrasynaptic cleft where bond to the N-terminal domain of LRP4 which activate LRP4/MUSK pathway and induces NMJ differentiation (Wu, Xiong, & Mei, 2010). Interestingly, muscle fibres of mice with mutation in agrin gene (agrin<sup>-/-</sup>) have no NMJ and ACHR are distributed along the defected muscle fibre. However, mutant mice are able to form clusters of ACHR suggesting that agrin might not be crucial for development of NMJ *de novo* (Wu, Xiong, & Mei, 2010).

The main organizer of clustering of the ACHR before innervation is Musk. Musk extracellular region is composed of Frizzled-like domain that is one of the cysteine rich domains that is activated either by agrin or WNT proteins (Messeant et al., 2015). In Musk<sup>-/-</sup> muscle fibres, ACHR are localized evenly along the fibre and Musk<sup>-/-</sup> mice do not form ACHR clusters (Wu et al., 2010). Musk is a tyrosine kinase receptor and therefore interacts with abundance of proteins that modulates activity and so its function is not dependent only from activation by agrin (Wu et al., 2010).

The connection between the two crucial proteins agrin and Musk is the single – pass transmembrane protein low-density lipoprotein receptor-related protein 4 (Lrp4). Lack of this protein results in death of the organism with NMJ appearance as in Musk<sup>-/-</sup> mice. LRP4 is co-receptor of Musk activated by agrin for ACHR clustering. Furthermore, co-localization of Musk/LRP4 receptor in the membrane determines position of postsynaptic domain and attraction of the axon cone (Happe, Tenerelli, Gromova, Kolb, & Engler, 2017).

Last but not least, glial neurotrophic derived factor (GDNF) has been proven as the most potent factor for motoneuron survival *in vitro*. Muscle cells produce GDNF and the receptor is located at the nerve endings of motor neurons. Addition of GDNF to cell culture exhibit in higher motor neuron survival and increased frequency and strength of muscle contractions (Zahavi et al., 2015). Furthermore, Wang et al. in 2002 using *Xenopus* nerve – muscle co-culture demonstrated not only that GDNF significantly increased total length of the neurons, number and size of the transmitting vesicles but also the size of acetylcholine receptor has been enlarged. Also, overexpression of GDNF in transgenic mice or GDNF injections results in numerous innervation and delay degradation of functional synapse *de novo* (Wu, Xiong, & Mei, 2010). Therefore, GDNF probably acts in retrograde manner and might regulate presynaptic as well as postsynaptic differentiation of neuromuscular junction (Wang et al. in 2002).

#### FUNCTION OF NMJS

NMJs are neural synapses responsible for transduction of signals between the motor nerve terminal and skeletal muscle fibres that subsequently generate muscle contraction. The synapses are composed of 3 major components: pre – synaptic motor nerve terminal, intra – synaptic cleft and post – synaptic basal lamina of muscle (Gonzalez-Freire et al., 2014).

The mechanism responsible for signal transduction involve specific molecules and is precisely orchestrated. After action potential reaches axon terminal, voltage-gated calcium channels open and influx of calcium ions occurs into the nerve terminal. Calcium ions bind to vesicles containing neurotransmitter – acetylcholine (ACH). Afterwards, vesicles move towards cell membrane, fuse with the membrane of axon terminal and release ACH to synaptic cleft by exocytosis. Subsequently, after ACH reaches nicotinic acetylcholine receptors (nACHR) conformational change opens sodium channels located on muscle membrane and sodium ions influx begins into the cell. Once the depolarization of the membrane reaches its threshold, voltage – gated calcium channels opens and cause sarcoplasmatic reticulum to release even more calcium that is propagated through gap junctions to nearby muscle cells. Therefore, action potential is spread across the whole muscle and contraction can occur. After the signal

is delivered, enzyme acetylcholinesterase is released from postsynaptic membrane and breaks down the excess of ACH that remains in the synaptic cleft to prevent prolonged or repetitive stimulations of ACHRs. The defect or degeneration can befall at any of these 3 components – presynaptic, intrasynaptic or postsynaptic (Goals, 2008).



Figure 2:Molecular basis of muscle contraction. Role of NMJs. Action potential travels from MNs body to presynaptic part of the NMJ and ACHs are released to the synaptic cleft where bind to ACHRs. Subsequently calcium is released from sarcoplasmatic reticulum and contraction can occur.

#### DEGENERATION OF NMJS AND ASSOCIATED DISEASES

Degeneration of neuromuscular junction is part of several conditions such as Amyotrophic Lateral Sclerosis (ALS), spinal muscular atrophy (SMA), major muscle trauma and aging. These conditions are characterized by NMJ disruption and motor neuron (MN) degeneration as the condition progresses. Treatment is currently limited and focused for extending life and alleviate from the symptoms (Happe et al., 2017). ALS is the most common disease affecting function of NMJ. Its origin is based in the genetics, where 2 major genes coding for C9orf72 and SOD1 are mostly defected (Perry, Han, Das, & Dickman, 2017). In 20 – 40 % of ALS patients C9orf72 gene contains hundreds to thousands of intron repetitions consisted of 6 nucleotides (GGGGCC<sub>n</sub>). This hexanucleotide repeats interfere with mRNA transcription and further producing not functional dipeptides that hampers other cellular functions (Perry et al., 2017).

Mechanism responsible for neural loss caused by aging still remains unclear. It may involve dysfunctional trophic signalling from both neurons or muscle cells, local degeneration of NMJ or damaged muscle fibres. However, the result of motor neuron denervation during aging could also be a result of lacking physical activity as person becomes older (Gonzalez-Freire et al., 2014). Unfortunately, once the motor neuron is lost, fibres that were controlled by this particular unit are no longer innervated and do not contribute to voluntary contraction of the whole muscle. Changes in the morphology include reduction of post-synaptic folds and nerve terminal area. Furthermore quantity of mitochondria in the myotube at the location of NMJ is significantly reduced, exhibiting disruption of cristae and fusing into megamitochondria. From degenerative point of view, any pathological alteration of the organelle providing energy and storing calcium ions may lead to disfunction of excite - contraction coupling and therefore degeneration of the NMJ. Morphologically or functionally hampered mitochondria can be found during aging in several tissues with increase oxidation or nitrosylation products. Since importance of properly functioning mitochondria increase with energy demands in the tissue also slight dysfunction may lead to major problems. In summary, impaired calcium release and ATP production in proximity of NMJ complicates transmission of the signal and vesicular reuptake (Rudolf, Khan, Labeit, & Deschenes, 2014). Another major problem of muscle weakness in progressed age is impaired function of sprouting and re – innervating denervated muscle fibres. Such a dysfunction leads to smaller motor units and weaker contractions what in final results in muscle fibres atrophy and transformation or reduction of type II fast glycolytic fibres (Gonzalez-Freire et al., 2014). Aging is closely connected with low grade inflammation. Interleukins 6 and 1, tumour necrosis factor alpha and C-reactive protein are inflammatory markers that are increased in circulating blood and tissues. Franceschi et al. (2007) composed term "inflammaging" by merging the terms together. Elevated levels of inflammatory

proteins in elderly leads to progressive loss of muscle mass and strength, disbalanced function of supporting cells and its secretion. Chronic inflammation is also connected with downregulation of IGF-1. In summary inflammation in elderly leads to problematic amino acid utilization and protein anabolism. At the presynaptic level we can observe tracks of oxidative stress, decrease number of synaptic vesicles and insufficient amount of released neurotransmitters (Gonzalez-Freire et al., 2014).

# POSSIBLE PREVENTION OF NMJ DEGENERATION

Caloric restriction (CR) and exercise have been described as possible interventions for NMJ degenerations. CR promotes preservation of many NMJs in the tibialis anterior muscle in mice observed for 24 months. NMJs of these mice exhibit reduced incidence of axonal atrophy and lower frequencies of fragmented or denervated postsynaptic sites. Another mechanism that is beneficial to NMJ preservation was upregulation of antioxidant manganese superoxide – dismutase that is responsible for elimination of superoxide produced in mitochondrial electron transport chain (Gonzalez-Freire et al., 2014). Exercise is another possibility to preserve NMJs in healthy condition. There is an evidence that physical activity postpone onset of aged – related muscle degenerations such as changed morphology or function (Rudolf et al., 2014). Furthermore GDNF protein content measured by enzyme linked immunosorbent assay have significantly increased in rats after 10, 20, 30 or 40 minutes of wheel running compared to age-matched controls (Gyorkos & Spitsbergen, 2014). However, exact mechanism behind the beneficial effect of exercise on NMJs preservation remains unclear.

## STATE OF ART OF CO - CULTURING

Synaptogenesis is challenging process to study *in vivo* in humans due to difficulties with spontaneous clustering and further synaptogenic differentiation. Explants of rat's phrenic nerve and diaphragm or another isolated innervated muscle strips that can bring light to development and metabolism of the NMJs are also not suitable for human NMJs investigation. Furthermore, ethical concerns about use of human embryogenic material prevents investigation of human development of NMJ and put in favour animals' models. The most feasible option to study NMJs are *in vitro* co-cultures of cells of different species in various arrangements (Mis et al., 2017).

Co-cultures are *in vitro* models based on culturing 2 or more different cell types in completely or partly common conditions and area. In case of NMJ, muscle cells and neural cells are cultured in many different *in vitro* models to identify and monitor NMJ formation/development. Both, neural and muscle compartments have several options to choose from. Explants from embryonic spinal cord, dissociated motor neurons, isolated ganglia, pheochromocytoma cells or neuroblastoma – glioma hybrid cells are just few options for neural component (Ostrovidov et al., 2017). The possibilities for skeletal muscle component involve muscle fibres, isolated myoblasts, differentiated satellite cells or embryonic skeletal muscle cells. Specific combinations and precise selection of both components affects afterwards the stages of NMJs development such as formation of basal lamina, ACHRs clustering and others (Mis et al., 2017).

Furthermore, in homologous models both components are from the same species, while heterologous are composed of components from different species. Many of the homologous or heterologous models has been described, however the most widely used contains random muscle compartment with embryonic rat spinal cord as neural component. For studying human NMJs, homologous model can be established using spinal stem cells derived from human foetus and primary skeletal muscle cells. Additionally, recent and promising human homologous model could be prepared from induced pluripotent stem cells derived to both neural and muscle component (Gajsek et al., 2008).

# AIMS AND OBJECTIVES

Primary and main objective was to create model of human neuromuscular junction *de novo in vitro* from primary myoblasts collected in surgeries / biopsies and induced pluripotent stem cells derived human motor neurons obtained from iXCells Biotechnology or isolated 7 days postnatal rat cerebellar granule neurons. Secondary objective was to prove positive effect on differentiation of myoblast and ACHR clustering of GDNF and agrin in culture.

# MATERIALS AND METHODS

## TISSUE COLLECTION AND CELL CULTURE

Muscle tissue was collected from anterior crucial ligament (ACL) surgeries as a waste tissue or muscle biopsies. Muscle tissue from surgeries was located on myotendinous junction from semitendinosus and gracilis muscle which was immediately put into the 50 ml falcon tube with 25 ml of PBS and 1% penicillin / streptomycin (Sigma-Aldrich, P4333). The tubes were then transferred to laboratory laminar airflow bench. Muscle tissue was dissected to 500 - 800 mg piece, cleaned from visible connective tissue, minced and added to tube with sterile filtered digestion medium (Promocell, C-23260; Roche Diagnostics A/S, 11088815001; Sigma-Aldrich, D4593-1G) and then placed for 1 hour into the incubator (Thermo Scientific, Forma Steri-cycle, CO<sub>2</sub> Incubator Model 317) at 37 °C, 5% CO<sub>2</sub>. Trituration was carried out every 15 minutes for better dissolvement. After digestion, 5 ml of culture medium (Promocell, C-23060; Promocell, C-39365; Sigma, G6784; Biowest, ALB – S1810) with 15 % FCS and 1 % L-Glutamine-Penicillin-Streptomycin was added to the suspension to stop the digestion and subsequently it was filtered through 100 µm cell strainer (BD Falcon, 352360) to remove debris. Another 5 ml of culture medium was used for washing the cell strainer. The suspension was centrifuged for 6 minutes at 600 g at room temperature. After centrifugation, supernatant was discarded and pellet was carefully resuspended in 1 ml of culture medium and divided to T25 flask which was then transferred to incubator at 37 °C and 5%  $CO_2$ . Medium was changed every 2 days. On the first change of the medium, old medium was centrifuged and the pellet was dissolved in 1 ml of new culture medium and returned to flask again. The cells were cultured for 7 days or to 80 % confluency when cell sorting takes place.

## IMMUNO AND MAGNETIC CELL SORTING

Medium from the flask was discarded and cells were washed twice with sterile PBS. Afterwards, cells were trypsinized with trypsin – EDTA solution (Biological Industries, 03-054-1B) until successful detachment and placed to incubator. To prevent damaging the CD56 antigen on the surface the time of trypsinization was limited to maximum of 2 minutes. Subsequently trypsin was inactivated by addition of culture medium (Promocell, C-23060; Promocell, C-39365; Sigma, G6784; Biowest, ALB – S1810) and

suspension of cells were transferred to a new tube and centrifuged for 6 mins at 600 g at room temperature. The pellet was then resuspended in 10 ml of PBS and cells were centrifuged again. Afterwards, newly formed pellet was resuspended with 170  $\mu$ l of MACS buffer (Milteneyi Biotec, cat. no. 130-091-221) (room temperature) and 35  $\mu$ l of CD56 magnetic beads (Miltenyi Biotec, cat.195 no. 130-050-401). The cells were incubated for 15 minutes at 5 °C in the fridge. After addition of 5 ml of MACS buffer, cells were again centrifuged for 6 mins and 600g. The pellet was then homogenized with 1 ml of MACS buffer. Hereafter, MiniMACS separator (Miltenyi Biotec, cat. no. 130-090-312) was placed to MultiStand magnet (Miltenyi Biotec, cat. no. 130-090-312) and Large Cell column (Miltenyi198 Biotec, cat. no. 130-042-202) with a 30 Pre-Separation filter (Miltenyi Biotec, cat. no. 130-041-199 407) was fixed onto the MiniMACS separator. The column was equilibrated by 500  $\mu$ l MACS buffer and cell suspension was added subsequently. While cells suspension was passing through column positive CD56 fraction was retained by the magnet while CD56 negative cells run freely and were collected to 15 ml tube. Three additional washes with MACS buffer of the column were performed to ensure complete wash out of unlabelled cells. Pre – separation filter was removed and 2,5 ml of MACS buffer was added to the column. The CD56 labelled cells were released by the pressure done with the piston and fraction was collected in new 15 ml tube. Into the both fractions 5 ml of culture medium was added and tubes were centrifuged as mentioned above. After centrifugation, cells were counted in Neubauer counting chamber (Labor Optik, 0.0025 mm<sub>2</sub>, depth 0.100 mm, cat. no. 1300000) and 20 000 cells of CD56 positive fraction were plated to 12 wells plate (Greiner Bio-One, cat. no. 665180).

## DIFFERENTIATION OF MYOBLASTS WITH AGRIN/GDNF ADDITION

Cell differentiation assay was performed. Sterilised glass coverslips (Marienfeld, 18 mm, cat. no. 0111580) were inserted to the 12 well plate and 1 ml of culture medium was added. Afterwards, 20 000 cells were seeded on the top of the coverslip and cells were left proliferating for 3 days. Medium was changed every other day. On day 3 culture medium was changed for differentiation medium containing Skeletal muscle basal medium (PromoCell, cat. no. C-23260,246 Heidelberg, Germany) and L-Glutamine-Penicillin-Streptomycin solution and cell was differentiating for 3,5 and 7 days. Firstly, according to literature (Ebrahimi et al., 2018; Ko et al., 2013), different concertation of agrin / GDNF (R&D Systems, 6624-AG-050, 212-GD-010) were used on differentiating myoblast. Tested concentrations were 10 ng/ml, 100 ng/ml and 1000 ng/ml. From the first day (day 3) of differentiation individual concentration of agrin / GDNF were added to the well and reintroduced when changing medium. Negative control was

performed alongside. After respective days in differentiation medium were coverslips processed for IHC or mRNA analysis.

## CO-CULTURING OF MYOBLASTS AND NEURONS

Myoblast were isolated as stated above and cultured for sufficient time to reach enough number of cells. Subsequently cells were trypsinized and 20 000 myoblasts were seeded on the top of the coverslips in culture medium in 12 well plate. Cells were proliferated for 3 days and then the medium was changed for either neural medium or myoblast differentiation medium and 20 000 cerebellar granule neurons (CGNs) were added. CGNs were provided by Stanislava Pankratova from the Department of Neuroscience, KU. These were isolated from day 7 Wistar rat pups. The cerebella were resected from rat pups, cleared from vessels and meninges and chopped. Hereafter the tissue was digested with trypsin, cell pellet was washed in DNAse I (Sigma-Aldrich, St. Louis, MO, USA) and soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA). After isolation, the cells were re-suspended in neurobasal medium (Gibco, Denmark) supplemented with 2 % (v/v) B27, 1 % (v/v) GlutaMax, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 20 mM HEPES (all from Gibco, Denmark) transferred to laboratory for plating. Co-culture was performed for 1 and 2 days. Triplicates for either immunostaining or qPCR analysis were carried out for every condition. Negative controls were performed alongside. All plates were kept in the incubator at 37 °C, 5% CO<sub>2</sub>.

#### FIXATION AND IMMUNOCYTOCHEMISTRY

Fixation was carried out with Histofix (Histolab, cat. no. 01000). Cell were first washed with PBS and adequate volume of Histofix was added. Cells were kept at room temperature for 8 minutes when cultured alone and 20-30 minutes when co-cultured with neurons. After fixation Histofix was aspirated from the wells and cells were washed 3 times with PBS and then kept in the PBS at 5 °C until immunocytochemistry. Firstly cells were permeabilized with 500  $\mu$ l of 0,01 % Triton-X solution (Sigma-Aldrich, cat. 301 no. 9002-93-1) in 0,05 M Tris Buffered Saline (TBS) for 8 minutes at room temperature. Secondly primary antibodies mixture (see Table 1) in blocking buffer (1 % Bovine Serum Albumin (Sigma-Aldrich, cat. no. A3912-100G), 0.1 % sodium azide (Sigma-Aldrich, cat. no. S-304 2002) in TBS) were added to the wells and incubated overnight at 4 °C. Next day, cells were washed with TBS and secondary

antibody mixture in blocking buffer were added to the wells and incubated for 1 hour at room temperature in dark. Alpha-bungarotoxin conjugated with AF488 was added to secondary antibodies mixture when needed. After incubation cells were washed with TBS and coverslips were mounted on the glass slides with a drop of Prolong Gold antifade reagent containing DAPI (Invitrogen, cat. no. P36931). At last, slides were kept overnight in the dark to dry completely and stored in – 20 °C or observed following day at fluorescent microscope (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany).

Primary antibodies							
Host	Antibody	Concetration	Company	Cat. no.			
Rabbit	Desmin	1:1000	Abcam	AB32362			
Mouse	TE7	1:100	Millipore	CBL271			
Rabbit	bit GAP43 1:1000 Millipore AE						
Secondary antibodies							
Rabbit	568 (Red) IgG	1:500	Invitrogen	A11036			
Mouse	488 (Green) IgG	1:500	Invitrogen	A11029			
Toxins							
Alpha-bungarotoxin	488 (Green)	1:100	Invitrogen	B13422			

Table 1: Antibodies and toxins used for immunocytochemical staining.

## MRNA EXTRACTION

After finished differentiation protocol or co-culture protocol, coverslips were moved to the new wells and 1 ml of TriReagent (Molecular Research Inc., cat. no. TR118) was added to each well and resuspended 20 times. Suspension was then moved to 2 ml a Biospec sterile tube (Bio Spec Products Inc., cat.265 no. 5225) and kept at - 80 °C until further analysis.

Continuing with the extraction of mRNA 100  $\mu$ L bromo-chloropropane was added to the TriReagent and left at room temperature for 15 min. Hereafter spun at 12,000 g for 15 minutes in order to separate the sample into an aqueous (containing RNA) and an organic phase. To precipitate the RNA from the aqueous phase (350  $\mu$ l) 80  $\mu$ g glycogen (Invitrogen, cat. no. 10814-010) and 350  $\mu$ L isopropanol were added, mixed and left for 10 minutes at room temperature. Hereafter spun 12,000 g for 8 minutes in room temperature. The RNA pellet was washed first with 1 mL 75 % ethanol, spun at 7,500 g for 5

minutes twice, removing the supernatant in between and dissolved in 100  $\mu$ L RNase-free water. Then re-precipitated with 10  $\mu$ L 3 M sodium acetate, pH 5.5 and 200  $\mu$ L of 99 % ethanol applying the same washing procedure as above. And lastly, the pellet was dissolved in 10  $\mu$ L RNase-free water. Total RNA concentration and purity were determined by spectroscopy with RiboGreen RNA assay Kit from 1  $\mu$ l RNA (Thermo Fisher, R11490).

## REAL-TIME PCR

100 ng RNA was converted to 20 µL cDNA using OmniScript reverse transcriptase (Qiagen, California, USA) and 1 mM poly-dT according to the manufacture's protocol (Qiagen). 0.25 µL cDNA was amplified in a 25 µl SYBR Green polymerase chain reaction (PCR) containing 1 × Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer for every target mRNA (see Table 2). A MX3005P Real-time PCR machine (Stratagene, California, USA) was used for monitoring the amplification, and a standard curve was made with known concentrations of the cloned PCR products or DNA oligonucleotides (Ultramer oligos, Integrated DNA Technologies, Inc., Leuven, Belgium) including a DNA sequence corresponding to the expected PCR product. The Ct values were related to the standard curve. Melting curve analysis after amplification was used to confirm the specificity of the PCR products and RPLPO mRNA was chosen as internal control. To support the use of RPLPO, another unrelated "constitutive" mRNA, GAPDH, was measured, and normalized with RPLPO. All targets were normalized to RPLPO. PCR primers can be seen in the Table 2.

Human			
Target	Primer name	Sense	Anti-sense
RPLPO	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
Myogenin	NM_002479.5	CTGCAGTCCAGAGTGGGGCAGT	CTGTAGGGTCAGCCGTGAGCAG
CHRNA1	NM_000079.3	GCAGAGACCATGAAGTCAGACCAGGAG	CCGATGATGCAAACAAGCATGAA
CHRNB1	NM_000747.2	TTCATCCGGAAGCCGCCAAG	CCGCAGATCAGGGGCAGACA
CHRND	NM_000751.2	CAGCTGTGGATGGGGCAAAC	GCCACTCGGTTCCAGCTGTCTT
CHRNE	NM_000080.4	TGGCAGAACTGTTCGCTTATTTTCC	TTGATGGTCTTGCCGTCGTTGT

CHRNG	NM_005199.5	GCCTGCAACCTCATTGCCTGT	ACTCGGCCCACCAGGAACCAC
MUSK	NM_005592.3	TCATGGCAGAATTTGACAACCCTAAC	GGCTTCCCGACAGCACACAC
Ki67	NM_002417.4	CGGAAGAGCTGAACAGCAACGA	GCGTCTGGAGCGCAGGGATA
Rat			
RPLPO	NM_022402.2	CCAGAGGTGCTGGACATCACAGAG	TGGAGTGAGGCACTGAGGCAAC
GAPDH	NM_017008.4	CCATTCTTCCACCTTTGATGCT	TGTTGCTGTAGCCATATTCATTGT

Table 2: RNA primers used in qPCR analysis of the experiments.

## IMAGE ANALYSIS

Images were taken by digital camera (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany) mounted on an Olympus BX51 microscope, using a 10x and 20x objective and using the software cellSens Standard 1.14 (Olympus, Olympus Soft 325 Imaging Solutions, GmbH, Münster, Germany). 3 images were blindly taken from different position and the most representative were chosen for figures. For analysis of purity of MACS 3 images were taken from respective sortings (in total 9 images) and analysed in FIJI version 2.0.0-rc-69/1.52n by custom macro as well as manually. Purity was identified as ratio of desmin positive nuclei to all nuclei. Number of all nuclei varied from 300 – 1000 per image. The quantification of the length of the neurites and branching was calculated by stereological means using software ProcessLengths (Protein Laboratory, University of Copenhagen). The method lays in counting every intersection of the processes and the grid (fig 3). Number of the intersection is afterwards related to the cell bodies. 1<sup>st</sup> branching is calculated as number of branches separated from main neurite related to the cell bodies.



Figure 3: Quantification of the processes in the ProcessLengths program. Neural bodies are marked with purple dots and every intersection between the grid and neurite is marked with orange colour. Slashed line is not included in the counting.

## STATISTICAL ANALYSIS

Statistical analysis was performed using unpaired Student T – test. Data were analysed by GraphPad software (version 8, GraphPad Software Inc., La Jolla, California). Data are presented as mean ± standard deviation (SD).



Figure 4: Overview of the experiments. Digestion of the tissue from the waste surgery / biopsy has been sorted and seeded to the flask. In the 1st experiment concentration of agrin were tested and evaluated by ICC. 2nd. and 3rd experiment was designed for observation of ACHR clustering in enriched culture with agrin or GDNF (100 ng/ml). Assessment was done by ICC and qPCR. 4th experiment was to established co-culture of human myoblasts and rat CGNs.

# RESULTS

#### MACS EXHIBITS SUFFICIENT PURITY OF CD56+ CELLS

Cell sorting efficiency was over 90 % of CD56+ cells in every tested sample (fig. 5). Highest purity of CD56 positive cells was in last tested sorting with purity of 95,86 % and lowest purity in first tested sorting with 92,02 % of CD56+ cells (fig. 5b). There was no considerable difference between respective MACS. In figure 5a we can see, that CD56+ cells, considered as myoblast/myotubes have elongated shape with multiple nuclei within one myotube. On the other hand, cells that are positive for fibroblast marker TE7 are more rounded, triangle shaped with one nuclei.



Figure 5: (a) Immunocytocemistry of magnetic cell sorting with myoblast marker CD56 conjugated with magnetic beads. Stained myoblast (desmin, red) with multiple nuclei (DAPI, blue) and single nucleated fibroblast (TE7, green). Scale bar equals 200  $\mu$ m. (b) Relative number of CD56+ cells in 3 independent performed MACS. Bars represent standard deviation.

Tested concentration of agrin has not shown any differences in myoblast differentiation (as assessed subjectively) and no signal from ACHRs (alpha-bungarotoxin) were recorded. Myotubes cultured with different concentration of agrin looked very alike, with long spindle like shaped forms with branching and having multiple nuclei that were aligned (fig. 6).



Figure 6: Tested concentrations of agrin on myoblast differentiation. Visually no signal was recorded from alpha-bungarotoxin (BTX, green). Aligned nuclei (asterix) are benchmark of differentiated myotubes. Fused myoblast can be seen on desmin channel (head arrow). Scale bar equals 200 µm.

## AGRIN HAS NO EFFECT ON MYOTUBE DIFFERENTIATION AND ACHR CLUSTERING

According to ICC, in myoblast culture with addition of 100 ng/ml of agrin, there was no positive signal from alpha-bungarotoxin conjugated with AF488 compared to control (fig.7a). Maturity of myotubes has not been improved alongside. In figure 7a, it can be seen that myotubes formed in both conditions

are elongated with multiple nuclei and no major difference can be seen between corresponding days. Myotubes formed at day 5 looks larger with more nuclei fused in one myotube than at day 3 in both conditions. At day 7, it can be observed that the number of myotubes decreased profoundly and this tendency can be seen also at day 9 of differentiation (fig 7a,b,c; supplementary fig 1 and 3). Furthermore, the shape of the myotubes at day 9 has become fragmented. According to qPCR results (fig 7a,b,c), the relative expression of genes tested had decreasing tendency over time and just slight difference can be seen between treatment group and control. From all tested genes, ACHR isoforms as well as MUSK were expressing similar pattern with slight decrease of expression at day 7 and with major



Figure 7: Tested concentration 100 ng/ml of agrin on myoblast differentiation. Visually no signal was recorded from alpha-bungarotoxin (BTX, green). Scale bar equals 200  $\mu$ m. (a) ICC of the respective days and conditions. (b,c,d) qPCr results presented as relative expression of 3 genes – MUSK, CHRNG, CHRND.

The results from mRNA analysis have shown that GDNF might contribute to ACHR clustering, mostly for isoform of the CHRNG, CHRND of the receptor. MUSK and other tested genes display similar pattern, however to lesser extent (fig 8b,c,d). It can be seen that relative expression increase over time until day 5 and subsequently drops thoroughly. Furthermore cell treated with 100 ng/ml of GDNF exhibit higher relative expression of the isoform of ACHR. From figure 8a it can be seen that addition of GDNF increase fusion of the myoblasts to myotubes. In both conditions massive myotubes can be observed with several nuclei, however myotubes with GDNF were interconnected and larger in size. Overtime myotubes stretched so it can be seen that at day 5 myotubes are less thick and more elongated (fig 8a, supplementary fig 3). (a)



0.0

3(a)

3(b)

5

Days in culture

7

equals 200  $\mu$ m. (a) ICC of the respective days and conditions. (b-d) qPCr results presented as relative expression of 3 genes – MUSK, CHRNG, CHRND.

# NEURONS CREATE LONGER AND MORE NEURITES IN CO-CULTURE AND NEURAL MEDIUM

Seeded CGNs differentiated better in the neural medium when co-cultured compared to control. Furthermore when co-cultured, neurons creates longer processes and have more branches in muscle differentiation medium (fig.9, 10). However, from ICC no visible alpha-bungarotoxin signal was present in neither conditions. Myotubes were more maturely formed in the muscle differentiation medium than in neural medium, however some myotubes were also observed in neural medium (fig. 9). Neurons were localized close to the newly formed myotubes, more often laying on the top rather than attaching to the space between the myotubes (fig. 9). Some clusters of neural cells were also created. qPCR results showed below, predicts interaction of neurons and myoblast, as all the detected genes are expressed less than in myoblast only control (fig. 11). This was also confirmed by phase – contrast microscopy (Supplementary fig. 4).



Figure 9: ICC of the co-cultured myoblasts and CGNs for one day in two respective media. Neuron control as well as myoblast control were performed in parallel.



Figure 10: Assessment of differentiation of CGNs in co-culture in two respective media. MM – myoblast differentiation medium, NM – neural basal medium, NO – control when neurons were seeded alone in both media.



Figure 11: qPCR results of 3 genes CHRNB1, CHRNE and MUSK. These genes are present in development of NMJ. MM – myoblast differentiation medium, NM – neural basal medium. Co – culture represents myoblasts and CGNs. Control – only myoblasts cultured in respective media.

## DISCUSSION

Sarcopenia is a condition defined by loss of mass, function or strength of the skeletal muscles mostly due to aging, physical inactivity or due to muscle affecting diseases such as ALS. Patients with sarcopenia are physically less mobile, have poor quality of life what might lead to worsening of the overall condition and other comorbidities even death (Santilli, Bernetti, Mangone, & Paoloni, 2014). NMJ degeneration is contributing factor of sarcopenia and is thoroughly studied through plentiful animal models co-cultures (Rudolf et al., 2014). Nonetheless, establishment of human model has not been still successful because of complications on molecular, material and ethical matter (Slater, 2017). On molecular level, there are several molecules such as agrin and GDNF that play crucial role in formation of NMJ. These molecules are secreted by cells in proximity of NMJ, secreted to the intrasynaptic space and transferred in anterograde as well as retrograde manner (Zahavi et al., 2015).

In the presented study, we tried to establish a model of co-culture of human neural cells and human primary myoblasts. Furthermore different concentration and length of differentiation of agrin and GDNF were tested. Primary myoblasts were obtained from waste surgeries / biopsies and sorted by MACS. Purity of the CD56+ fraction was over 90 % in all tested sorting. Sufficient purity is important for studying interactions between two cell types that are co-cultured.

In the first experiment different concentrations of agrin have been tested on differentiating myoblast for 3 days. Assessment from ICC has shown that no signal from alpha-bungarotoxin was present and shape of the myotubes was no different from control at any concentration of agrin. Chang et al., have shown that 0,1  $\mu$ g/ml concentration of agrin added to C2C12 culture overnight is sufficient for acetylcholine clustering (Chang et al., 2012). In other study, that also focused on alignment of rats myotubes and expression of differentiation markers as myogenin showed that addition either 10 or 100 ng/ml of agrin to the culture in first day of differentiation has significant impact on myotube maturation (Ebrahimi et al., 2018). According to the results and literature review, concentration of 100 ng/ml has been chosen for further experiments with potential effect on ACHR formation.

Referring to Mis et al., different species has various times for ACHRs to form cluster and create NMJ synapse. For this reason, several time points has been chosen in third and fourth experiment with addition of 100 ng/ml of either agrin or GDNF. Results showed that while agrin has had no effect on myoblast differentiation over time, myoblast cultured in GDNF enriched medium exhibit better fusion and differentiation according to ICC. This was also confirmed by qPCR results that showed that there was some elevation in expression of ACHR isoforms CHRNG, CHRND and MUSK in GDNF experiment

from day 3 until day 5. In agrin experiment the genes were downregulated over time. Both experiments showed larger decrease after 5 days of differentiation probably because of detachment of myotubes from glass coverslips and dying. GDNF is produced by myoblast and acts as an attraction molecule for neurites to create intrasynaptic connection – NMJ (Wang et al., 2002). Presented results indicate that GDNF delivered externally might interact with myoblasts and improve differentiation capacity as well as gene upregulation of ACHR *in vitro*. Similar model could work also as autoregulation as a response to stimulus such as exercise or caloric restriction.

Finally, co-culture of human myoblast and rats CGNs was performed. It is positive that in both tested media the cells differentiate and habituate. Neurons were mostly located on the myoblasts or very close to them. Probably myoblast produced some extracellular matrix that could serve as attachment for neurons (Beach, Rao, & Festoff, 1985). Neurons attachment should be tested on coverslips coated with Matrigel and compared to not coated control. Neurites were longest In the neural medium when co-cultured with myoblast compared to neuron only control. Moreover, long neurites were abundant also in myoblast medium when co-cultured. From qPCR results it can be assumed that there is interaction between the co-cultured cells. Expression of the ACHR isoforms and MUSK genes was lower than in myoblasts only control. Limitation of rats' CGNs are their short lifetime (1-3 days in culture) that might be insufficient for human NMJ to develop. No data are presented from day 2 of differentiation because of contamination of the plates.

After these pilot experiments, it was proceeded to final experiment, where 5 donors of myoblast were chosen of different age and seeded in triplicates to 12 well plates. Co-culture was repeated with 2 different rats donors of neurons. Unfortunately the data has not been analysed yet and therefore cannot be shown in the thesis.

On the one hand human myoblast and rat co-culture and its following qPCR analysis, partly uncovered interaction between the cells and GDNF influence of myoblast are considered as interesting findings of this research. On the other hand, presented experiments has many limitations. One of the crucial limitation is small number of samples in respective experiments and therefore lacking statistical analysis. Nevertheless experiments were pursued as pilot tests for further navigation in creation of model of cell co-culture. Also throughout the study, contamination was experienced as a results of bad manipulation with the waste surgery during the transfer and sometimes poor laboratory practice.

Investigation of NMJ can lead to better understanding of its role in sarcopenia and related diseases. Step by step approach could be beneficial in creation of human homologous model. Furthermore addition of human motor neurons to partly differentiated myotubes might has beneficial effect for NMJ formation. In the future it would be very interesting to investigate also differences of subjects that are physically active to those that are not, age differences, sex differences or administration of specific molecules such as agrin / GDNF to subjects or to culture. Physical exercise as an external stimulus could be very beneficial for preventing of NMJ loss. It was proven in mice and rats that underwent endurance as well as resistance training had hypertrophy in NMJ areas and higher secretion of ACH. In contrast lower physical activity results in NMJs degeneration and terminal sprouting of the MNs (Nishimune, Stanford, & Mori, 2014). Stimulus from exercised muscle induce recruitment of macrophages to damaged tissue, satellite cell activation and upregulation of several genes. Stimulated myoblast secrete neurotrophic factors such as neurotrophin 4, insulin-like growth factor-1, brain-derived neurotrophic factor. These are subsequently secreted to the intrasynaptic cleft and retrogradely transported to MN's cell body (Mills et al., 2018; Wu et al., 2010; Zahavi et al., 2015).

Nowadays it is also possible to co-culture cells in special partly isolated compartment thanks to microfluidic devices or inserts that have been already tested on mice myoblasts. The principle is based on two separated compartments that are connected with microchannels where exchange of molecules and neurite outgrowth is possible (Blizzard et al., 2015; Happe et al., 2017; Mills et al., 2018; Sackmann, Fulton, & Beebe, 2014). In our study we tested the chip for myoblasts attachment, but because of limited source of neurons classic co-culture was used for its simplicity. However it would be beneficial to involve this technology in future studies.

# CONCLUSION

Current research of NMJs degeneration is investigated mostly through *in vitro* animal models. In the presented study heterologous human-rat co-culture was developed and effect of agrin and GDNF on myoblast differentiation and ACHR clustering was observed. Neural cells and myoblast co-cultured exhibit some form of interaction according to ICC and qPCR results. Furthermore myoblasts cultured with addition of GDNF display higher expression of genes closely connected to ACHRs. On the contrary, different concentration of agrin has no effect on the cultured myoblasts as well as length of exposure to the molecule. Unfortunately, major drawback of this study is its lack of repeated data. However, these pilot experiments were valuable in designing future advance in research of NMJ. The data are not included due to shortage of time and analysis. Finally, further research of NMJs degeneration is basically dependent in development of homologous *in vitro* model of NMJ that optimal mimics *in vivo* conditions.

# ACKNOWLEDGMENT

Firstly, I would like to thank to my supervisor Abigail Mackey and Cristian Pablo Pennisi for their kind guiding and valuable advices throughout the elaboration of the thesis. Secondly, many thanks are given to Stanislava Pankratova from Department of Neuroscience, KU for providing CGNs. I am also grateful for Anja Jokipii-Utzon, Ann – Christina Ronnié Reimann, Chloé Yeung, Monika Lucia Bayer and Casper Sønderbroe for their help in the laboratory. I would like to thank Peter Schjerling for the help with analysing mRNA data. Last but not least, I am thankful for my family and Barbora for their patience, love and support throughout my whole studies.

# BIBLIOGRAPHY

- Beach, R. L., Rao, J. S., & Festoff, B. W. (1985). Extracellular-matrix synthesis by skeletal muscle in culture, 225, 619–627.
- Blizzard, C. A., Southam, K. A., Dawkins, E., Lewis, K. E., King, A. E., Clark, J. A., & Dickson, T. C. (2015). Identifying the primary site of pathogenesis in amyotrophic lateral sclerosis - vulnerability of lower motor neurons to proximal excitotoxicity. *Disease Models & Mechanisms*, 8(3), 215–224. https://doi.org/10.1242/dmm.018606
- Ebrahimi, M., Ostrovidov, S., Salehi, S., Kim, S. B., Bae, H., & Khademhosseini, A. (2018). Enhanced skeletal muscle formation on microfluidic spun gelatin methacryloyl (GelMA) fibres using surface patterning and agrin treatment. *Journal of Tissue Engineering and Regenerative Medicine*, 12(11), 2151–2163. https://doi.org/10.1002/term.2738
- Gajsek, N., Jevsek, M., Mars, T., Mis, K., Pirkmajer, S., Brecelj, J., & Grubic, Z. (2008). Synaptogenetic mechanisms controlling postsynaptic differentiation of the neuromuscular junction are nerve-dependent in human and nerve-independent in mouse C2C12 muscle cultures. *Chemico-Biological Interactions*, 175(1–3), 50–57. https://doi.org/10.1016/j.cbi.2008.05.027
- Goals, P. (2008). The Neuromuscular Junction, 91, 6-11.
- Gonzalez-Freire, M., de Cabo, R., Studenski, S. A., & Ferrucci, L. (2014). The neuromuscular junction: Aging at the crossroad between nerves and muscle. *Frontiers in Aging Neuroscience*, 6(AUG), 1–11. https://doi.org/10.3389/fnagi.2014.00208
- Gyorkos, A. M., & Spitsbergen, J. M. (2014). GDNF content and NMJ morphology are altered in recruited muscles following high-speed and resistance wheel training. *Physiological Reports*, *2*, e00235. https://doi.org/10.1002/phy2.235
- Happe, C. L., Tenerelli, K. P., Gromova, A. K., Kolb, F., & Engler, A. J. (2017). Mechanically patterned neuromuscular junctions-in-a-dish have improved functional maturation. *Molecular Biology of the Cell*, 28(14), 1950–1958. https://doi.org/10.1091/mbc.E17-01-0046
- Chang, Y. F., Chou, H. J., Yen, Y. C., Chang, H. W., Hong, Y. R., Huang, H. W., & Tseng, C. N. (2012). Agrin induces association of Chrna1 mRNA and nicotinic acetylcholine receptor in C2C12 myotubes. *FEBS Letters*, 586(19), 3111–3116. https://doi.org/10.1016/j.febslet.2012.07.068
- Ko, I. K., Lee, B. K., Lee, S. J., Andersson, K. E., Atala, A., & Yoo, J. J. (2013). The effect of in vitro formation of acetylcholine receptor (AChR) clusters in engineered muscle fibers on subsequent innervation of constructs in vivo. *Biomaterials*, 34(13), 3246–3255. https://doi.org/10.1016/j.biomaterials.2013.01.029
- Legay, C., & Mei, L. (2017). Moving forward with the neuromuscular junction. *Journal of Neurochemistry*, 142(Suppl 2), 59–63. https://doi.org/10.1111/jnc.14028
- Marty, E., Liu, Y., Samuel, A., Or, O., & Lane, J. (2017). A review of sarcopenia: Enhancing awareness of an increasingly prevalent disease. *Bone*, 105, 276–286. https://doi.org/10.1016/j.bone.2017.09.008
- Messeant, J., Dobbertin, A., Girard, E., Delers, P., Manuel, M., Mangione, F., ... Strochlic, L. (2015). MuSK Frizzled-Like Domain Is Critical for Mammalian Neuromuscular Junction Formation and Maintenance. *Journal of Neuroscience*, 35(12), 4926–4941. https://doi.org/10.1523/JNEUROSCI.3381-14.2015
- Mills, R., Taylor-Weiner, H., Correia, J. C., Agudelo, L. Z., Allodi, I., Kolonelou, C., ... Teixeira, A. I. (2018). Neurturin is a PGC-1α1-controlled myokine that promotes motor

neuron recruitment and neuromuscular junction formation. *Molecular Metabolism*, 7(November 2017), 12–22. https://doi.org/10.1016/j.molmet.2017.11.001

- Mis, K., Grubic, Z., Lorenzon, P., Sciancalepore, M., Mars, T., & Pirkmajer, S. (2017). In vitro innervation as an experimental model to study the expression and functions of acetylcholinesterase and agrin in human skeletal muscle. *Molecules*, 22(9). https://doi.org/10.3390/molecules22091418
- Nishimune, H., Stanford, J. A., & Mori, Y. (2014). Role of exercise in maintaining the integrity of the neuromuscular junction. *Muscle and Nerve*, 49(3), 315–324. https://doi.org/10.1002/mus.24095
- Ostrovidov, S., Ahadian, S., Ramon-azcon, J., Hosseini, V., Fujie, T., Parthiban, S. P., ... Ramalingam, M. (2017). Three-dimensional co-culture of C2C12 / PC12 cells improves skeletal muscle tissue formation and function, (November 2014), 582–595. https://doi.org/10.1002/term.1956
- Perry, S., Han, Y., Das, A., & Dickman, D. (2017). Homeostatic plasticity can be induced and expressed to restore synaptic strength at neuromuscular junctions undergoing ALSrelated degeneration. *Human Molecular Genetics*, 26(21), 4153–4167. https://doi.org/10.1093/hmg/ddx304
- Rudolf, R., Khan, M. M., Labeit, S., & Deschenes, M. R. (2014). Degeneration of neuromuscular junction in age and dystrophy. *Frontiers in Aging Neuroscience*, 6(MAY), 1–11. https://doi.org/10.3389/fnagi.2014.00099
- Sackmann, E. K., Fulton, A. L., & Beebe, D. J. (2014). The present and future role of microfluidics in biomedical research. *Nature*, 507(7491), 181–189. https://doi.org/10.1038/nature13118
- Santilli, V., Bernetti, A., Mangone, M., & Paoloni, M. (2014). Clinical definition of sarcopenia, *11*(3), 177–180.
- Shafiee, G., Keshtkar, A., Soltani, A., Ahadi, Z., Larijani, B., & Heshmat, R. (2017). Prevalence of sarcopenia in the world: A systematic review and meta- analysis of general population studies. *Journal of Diabetes and Metabolic Disorders*, 16(1), 1–10. https://doi.org/10.1186/s40200-017-0302-x
- Slater, C. R. (2017). The structure of human neuromuscular junctions: Some unanswered molecular questions. *International Journal of Molecular Sciences*, 18(10). https://doi.org/10.3390/ijms18102183
- Wang, C., Yang, F., He, X., Je, H., Zhou, J., Eckermann, K., ... Lu, B. (2002). Regulation of Neuromuscular Synapse Development by Glial Cell Line-derived Neurotrophic Factor and Neurturin \*, 277(12), 10614–10625. https://doi.org/10.1074/jbc.M106116200
- Wu, H., Xiong, W. C., & Mei, L. (2010). To build a synapse: signaling pathways in neuromuscular junction assembly. *Development*, 137(7), 1017–1033. https://doi.org/10.1242/dev.038711
- Zahavi, E. E., Ionescu, A., Gluska, S., Gradus, T., Ben-Yaakov, K., & Perlson, E. (2015). A compartmentalized microfluidic neuromuscular co-culture system reveals spatial aspects of GDNF functions. *Journal of Cell Science*, 128(6), 1241–1252. https://doi.org/10.1242/jcs.167544

# APPENDIX

# APPENDIX I - SUPLEMENTARY DATA



Supplementary figure 1:. Tested concentration 100 ng/ml of agrin on myoblast differentiation. Visually no signal was recorded from alpha-bungarotoxin (BTX, green). Only autofluorescence can be observed. Respective channels of day 3 and 5. Scale bar equals 200  $\mu$ m.



Supplementary figure 2:. Tested concentration 100 ng/ml of agrin on myoblast differentiation. Visually no signal was recorded from alpha-bungarotoxin (BTX, green). Only autofluorescence can be observed. Respective channels of day 7 and 9. Scale bar equals 200 µm.



Supplementary figure 3:. Tested concentration 100 ng/ml of GDNF on myoblast differentiation. Visually no signal was recorded from alpha-bungarotoxin (BTX, green). Only autofluorescence can be observed. Respective channels of day 3 and 5. Scale bar equals 200 µm.

# Myoblast differentiation medium

Neurobasal medium



Supplementary figure 4:. Phase-contrast microscopy of myoblasts co-cultured for one day in two respective media. Independently cultured neurons as well as myoblast were considered as control. Scale bar equals 200  $\mu$ m.

# Study 1, BBH protocol, Michal Tamas, 2019

Lab days

DAY 0: Cell thawing and culturingDAY 3: Cell sorting protocolDAY 7: Change to diff medium plus agrin / neurons.DAY 8: Fixation after one day. For PCR and IHC.

DAY 9: Fixation after two days. For PCR and IHC.

## Cell thawing and culturing

- Cells are taken out from freezer and thawed in water bath.
- Thawed cells are transferred to 15 ml tube with 5 ml of culture medium and put into the centrifuge (6min, 600g, RT).
- Pellet is resuspended in 1 ml of medium and transferred to T25 flask with 4 ml of culture medium.

# Cell sorting protocol \_\_\_\_/ at \_\_\_:\_\_

## Trypsinization

- Trypsin should be room temperature.
- Prepare a 15 mL falcon tube with 10 mL culture medium.
- Aspirate medium in the flask with the cells.
- Wash the cells twice with PBS (use 5 mL).
- Aspirate PBS.
- Add 1 mL 37°C warm 0.05% trypsin to the flask (T25) and look under the microscope to follow the loosening of the cells (heat in incubator of status quo).
- After 1-2 min all the cells should be loosen from the flask (bang on the side of the flask if not, to loosen the cells from the surface).
- Add 1 mL culture medium to the flask and mix. Pipette all the cells from the flask into the 15 mL falcon tube containing the prepared medium. This step should be done quickly since the trypsin can destroy the surface proteins on the cells, enable then to bind to the CD56 antibody used in the cell sorting protocol.
- Mix gently.
- Centrifuge for 6 min (600g).
- After centrifuge aspirate the supernatant and loosen the pellet on the grid in the bench.

## Cell sorting

- Heat culture medium in water bath beforehand.
- Add 10 mL of PBS to the cells.
- Centrifuge at 600 g for 6 min.
- Discard the supernatant.
- Homogenise the pellet with 170 μl of MACS buffer (room temp) and add 35 μl of CD56 magnetic beads.
- Incubate the cells with the magnetic beads at 5°C (in the fridge and not on ice) for 15 min.
- Add 5 mL MACS buffer.
- Centrifuge at 600g for 6 min.
- Discard the supernatant.
- Homogenise the pellet with 1 mL MACS buffer.
- Fix the MiniMACS separator on MultiStand magnet and put the column (Large cell column) on the MiniMACS separator. Add the Pre-Separation filter (yellow, 30 µm) on top of the column.
- Prepare two 15 mL falcon tubes: F- (to collect CD56- cells) and F+ (to collect CD56+ cells).
- Equilibrate the column with 500  $\mu$ L MACS buffer then add the cells. Collect the fraction under the column in the F- tube.
- Perform three washes with 1 mL MACS buffer and collect in the F- tube (the column must not dry between the washes).

- Remove the pre-separation filter.
- Add 2.5 ml of MACS buffer and immediately remove the column from the magnet.
- Flush the liquid inside the column with the piston in the F+ tube, in this way, you will recover all the cells bound to the CD56 beads that were retained in the column by the magnet.
- In the two fractions F+ and F-, add 5 ml of culture medium.
- Centrifuge the cells for 6 min at 600 g.
- Discard supernatant and resuspend the cells in 1 ml of culture medium.
- Count the cells in myoblast fractions and add x cells (dependent of the experiment) to each well on a 12well culture plate with a sterilized coverslip (See the counting protocol).
- The fibroblast fractions are frozen.

#### Counting cells

- Discard supernatant.
- Trypsinated cells are dissolved in 1 mL culture medium.
- Prepare Neubauer counting chamber.
- Add 10 ul to the counting chamber under the cover glass.
- Count the cells within the four big reg squares.
- Count all cells that are not touching the lower and the right border.
- Clean the Neubauer counting chamber.
- Calculate the average and multiply with 10.000. Divide target-cell-number with average cell count.
- Example: 128, 159 and 242 are counted. Average is 176. 176 x 10.000 = 1760000. 20000 / 1760000 = 0.011 mL.
- Therefore 0.011 mL / 11 ul should be added to each well for 20000 cells.

## Plating of cells

- Cells (CD56+) are then plated into 12 well plates containing proliferation conditions.
- Add 1 ml medium.
- Coverslip can be found in building. 8, but should be sterilized by Vibeke beforehand.
- Add coverslips to the wells on 6 x 12-well companion plate.
- Add cells (20000 (5000 cells/cm<sup>2</sup>)
- Leftover cells should be frozen down.. Spin the cells down and dissolve the pellet in 1 mL fridge cold freeze medium, and quickly add the 1 mL to a cryotube. Transfer it directly into the alcohol freeze box, then to 140°C freezer.
- Fill out plating page

# Changing to differentiation medium \_\_\_/\_\_\_ at \_\_\_\_ and \_\_\_/\_\_\_ at \_\_\_\_

- Heat medium in water bath beforehand.
- Change to either muscle differentiation medium (muscle basal medium) or neural medium on day 4.
- Add 10 ul of agrin / 20 000 neurons to wells.

Staining Histofix \_\_\_/\_\_\_ at \_\_:\_\_\_

- Take chamber out of the incubator and into fume hood.
- Aspirate medium.
- Add quickly fridge cold non sterile PBS, leave it for a minute, and mix gently.
- Remove PBS.
- Add 400 mL histofix until the cells are completely covered.
- Leave in the fume hood for 8 min without the lid.
- Remove the histofix.
- Wash thrice with PBS. Leave it for 1 min the first time. Leave it in the third time.
- Add once more PBS. Leave at 4°C until it's time to stain the slides.

Coverslip staining \_\_\_\_\_ at \_\_\_\_ and \_\_\_\_/\_\_\_ at \_\_\_\_

- Staining should be done as quickly as possible after the fixation
- When the coverslips are stained and put into the freezer then there no time-pressure on taking the images.

## Day 1:

- Prepare Triton solution and blocking buffer with antibodies (GAP43, MHCII). Use 500 ul per well. Always prepare extra.
- Wash with TBS
- Incubate with triton solution (300 ul) for 8 minutes.
- Wash with TBS
- Add blocking buffer with primary antibodies
- Cover the plate with tin foil and leave in the fridge O/N

Day 2:

- Prepare blocking buffer with antibodies (Mouse Mouse, Rabbit-rabbit when doing a double staining, alfa-BTX). Always prepare extra.
- Wash with TBS
- Add blocking buffer with secondary antibodies for 1 hour at room temperature covered in tin foil.
- Wash twice with TBS.
- Leave a small drop of mounting medium (DAPI) on a slide and put the coverslip on top with the cells downwards. Leave it to dry for 48 hours before transferring them to the -20°C freezer.

# mRNA extraction \_\_\_/\_\_\_ at \_\_\_\_ and \_\_\_/\_\_\_ at \_\_\_\_

- These steps are done in hood (Vibekes lab) at building 9. Bring tri reagent from RNA lab. Ask Anja for tubes.
- After finished cell culture protocol, move the coverslip to a new empty well (12 well plate) by using tweezers and a bended needle. Move one coverslip at the time.
- Add 1 mL tri reagent and mix by pipetting up and down a couple of time. The cells should be loosened instantly.
- Move the 1 mL Trireagent and cells to a 2 mL Biospec tube suited for mRNA extraction. These tubes should be labelled beforehand; but write the well number and date.
- Leave in the -80 °C freezer until further analysis.

## Plating

Differentiation (20000 cells per well)



1day



2days

	NM	MM	NM	MM	NM	MM	NM	MM	NM	MM	NM	MM
	Stain	Stain	mRNA	mRNA	Stain	Stain	mRNA	mRNA	Stain	Stain	mRNA	mRNA
Neurons+ Neurons-				NO	NO		NO	NO				

NO - neurons only in respective medium (NM,MM) MM – muscle medium NM – neural medium

One setup used for mRNA extraction and the same set up used for ICC (might be only duplicates in case of not enough cells). Total 4 plates.

## Formulas

**Culture medium (85% Human Skeletal Muscle Growth Medium, 15 % FBS, 0.4 % P/S)**: Add the supplement mix to the Skeletal Muscle Growth Medium (OBS expires after 6 weeks at 4-6 °C). Hereafter add 42.45 mL Growth medium to a 50 mL falcon tube, and add 7.5 mL FBS and 0.05 mL Glutamine P/S. Preheat the 50 mL falcon tube with the culture medium before use. (OBS mix the medium + supplement from old batch with new a new batch, 1:1)

**DIFF medium**: (Skeletal muscle basal medium and 0.1% L-Glutamine-Penicillin-Streptomycin solution), take 50 mL of the basal medium and add 50  $\mu$ l Glut/Pen/Step. Preheat before use.

**Freeze medium 20 mL:** Prepare 25 mL in a 50 ml tube. Use 10% Dimethyl Sulfoxide (DMSO) (2.5 mL) and 90% FBS (22.5 mL). Keep in fridge. Use 1 mL to disturb the pellet after centrifugation. Place in alcohol box and then in -160° freezer the day after. Use labels for cryotubes.

## 0.05 M Tris Bufferet Saltvand (TBS) (4 L) - Expires after two weeks:

400 mL Washing buffer from the fridge 3.6 L distilled water.

## Blocking buffer 50 mL tube (1 % BSA + 0.1 % sodium azide):

40 mL 0.05M TBS 0.4 g Albumin Bovine Serum (Sigma-Aldrich, ref:A3912-100G, lot: 071M1561V) 400 μl of 10 % Sodium Azide (Sigma-Aldrich, ref:S-2002)

## Triton solution (0.01% Triton in 0.05 M TBS)

50 mL 0.05 M TBS 50 μl Triton X-100 (Sigma-Aldrich, ref: 9002-93-1, x100-100 mL)

Products used Cryo-tubes 1.8 mL: Thermo Scientific ref: 377267, sterile Objectglass: Superfrost 76x26 mm (Hounisen ref: 1510.1205HV, 50 stk Tweezer

Trypsin: Gibco 0.25 % Trypsin EDTA (1x) ref:25200-056, lot:1368124. (Obs to get 0.13% trypsin dilute with PBS).

Neubauer improved counting chamber: Labor Optik ref: 1300000, 0.0025mm<sup>2</sup>, depth 0.100mm MACS running buffer: Milteneyi Biotec, ref:130-091-221 CD56 magnetic beads: Miltenyi Biotec, ref 130-050-401 MiniMACS separator: Miltenyi Biotec, ref:130-090-312 MultiStand: Miltenyi Biotec, ref:130-090-312 Large Cell Column: Miltenyi Biotec, ref:130-042-202, lot: 5131028006 Pre-separation filter 30 μm: Miltenyi Biotec, ref:130-041-407, lot:5130607101

DMSO: Sigma-Aldrich, ref: D2650, lot: RNBC1324 12-well companion plate: Corning, cat no: 353503 Coverslip 18mm: Marienfeld, ref:0111580, lot:26276819

Histofix: Histolab, ref: 01000, 1 L, Lot:151523 Trireagent (RNA/DNA/Protein Isolation Reagent): Molecular Research Inc., Cat no: TR118, Lot: 5021 Biospec tubes (Polypropylene microvials), sterile, 2 mL: Bio Spec Products Inc., Cat no: 5225

**Eppendorf tubes:** Safe-lock tubes 0.5 mL cat:0030 121 147 **DMSO:** Sigma-Aldrich, ref: D2650, lot: RNBC1324

Mounting medium: Prolong Gold antifade reagent with DAPI, Invitrogen, ref:P36931, lot:705131

Coverslip: 24 x 40 mm deckgläser, Hounisen

Glass staining plate: Marienfeld, ref: 1406809

#### Primary antibody used

Rabbit Desmin 1:1000 (BBH:PA271)(Y66, Abcam AB32362) Mouse TE7 1:100 (BBH:PA308 freezer)(Millipore, CBL271)

#### Secondary antibody used

Rabbit 568 (Red) IgG 1:500 (BBH:SA04)(Invitrogen, A11036) Mouse 488 (Green) IgG 1:500 (BBH:SA06)(Invitrogen, A11029) Mouse 568 (Red) IgG 1:500 (BBH:SA07)(Invitrogen, A11031)