A system to study mechanisms of neuromuscular junction development and maintenance

Valérie Vilmont*a, Bruno Cadot*a, Gilles Ouanounou and Edgar R. Gomesa,b*

a Myology Research Center, UM76-INSERM U974-CNRS FRE 361 Sorbonne Universités, UPMC Université Paris 06, Paris France

b Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

c FRE CNRS 3693 (U.N.I.C),Unité de Neuroscience, Information et Complexité CNRS, Bât. 33, 1 Ave de la Terasse, 91198, Gif sur Yvette, France

d Cochin Institute, Transmission Electron Microscopy Platform, INSERM U1016, CNRS UMR8104, Paris Descartes University Sorbonne Paris Cité, Paris, France.

Authors’ email addresses:
Bruno Cadot: cadotbruno@gmail.com
Gilles Ouanounou: gilles.ouanounou@unic.cnrs-gif.fr

*Corresponding authors:
Dr. Valérie Vilmont
Myology Research Center, UM76-INSERM U974-CNRS FRE 361 Sorbonne Universités, UPMC
Université Paris 06, Paris France
Email: vilmont.valerie@gmail.com

Dr. Edgar Gomes
Instituto de Medicina Molecular
Faculdade de Medicina da Universidade de Lisboa
Av. Professor Egas Moniz
1649-028 Lisboa
Portugal
Phone: +351 217999515
Fax: +351 217 999 412
Email: edgargomes@medicina.ulisboa.pt

Keywords: co-culture, differentiation, myofiber, NMJ
ABSTRACT

The neuromuscular junction (NMJ), a cellular synapse between a motor neuron and a skeletal muscle fiber, enables the translation of chemical cues into physical activity. The development of this special structure has been subject to numerous investigations, but its complexity renders in vivo studies particularly difficult to perform. In vitro modelling of the neuromuscular junction represents a powerful implement to fully delineate the fine tuning of events that lead to subcellular specialization at the pre-synaptic and post-synaptic sites. Here we describe a novel heterologous co-culture in vitro method using rat spinal cord explants with dorsal root ganglion and murine primary myoblasts to study neuromuscular junctions. This system allows the formation and long-term survival of highly differentiated myofibers, motor neurons, supporting glial cells and functional neuromuscular junctions with post-synaptic specialization. Therefore, fundamental aspects of NMJ formation and maintenance can be studied using the described system which can be adapted to model multiple NMJ-associated disorders.
INTRODUCTION
The continuous development of new experimental approaches have proved beneficial to model a number of adverse health conditions among which neuromuscular diseases (Chew et al., 2015; Lenzi et al., 2015; Sandoe and Eggan, 2013; Tu et al., 1996). Neuromuscular pathologies encompass a wide-range of sub-groups among which (i) myopathies like Duchenne's and Becker's muscular dystrophies (Flanigan, 2014), (ii) motor-neuron diseases (MNDs) like amyotrophic lateral sclerosis (ALS), progressive bulbar palsy, pseudobulbar palsy and spinal muscular atrophy (SMA) (de Boer et al., 2014; Edens et al., 2015; Karam et al., 2010; Tu et al., 1996) and (iii) auto-immune neuromuscular diseases like myasthenia gravis and Lambert Eaton myasthenic syndrome (Ha and Richman, 2015; Lang et al., 2003).

Researchers have set up different animal models and cell lines (Chen et al., 2014; Corti et al., 2012; Lenzi et al., 2015) with the hope of recapitulating some features of the latter group and understand the triggers of one of their common hallmarks: the disruption of the neuromuscular junction (NMJ). The NMJ is one of the most studied synapses. It is formed of three key elements: the lower motor neuron (the pre-synaptic compartment), the skeletal muscle (the post-synaptic compartment) and the Schwann cell (Sanes and Lichtman, 1999). The NMJ is formed in a step-wise manner following a series of cues involving these three cellular components and its role is basically to ensure the skeletal muscle functionality. Following an action potential down the motor neuron axon, synaptic vesicles will fuse with the membrane at the half-terminal of the axon releasing neurotransmitters in the synaptic cleft. The post-synaptic membrane of the muscle fiber is specialized to respond efficiently to the neurotransmitter release and will convert the chemical signal to a mechanical signal in the form of muscular contraction (Das et al., 2010). In some cases, the dialogue between these cellular components is compromised and leads to the instability of the NMJ and in worst cases like in ALS and SMA, eventually axon retraction and muscle atrophy.
In order to study NMJ physiology and pathology, different \textit{in vivo} systems are used such as mouse diaphragm or drosophila abdominal segments (Packard et al., 2002; Perez-Garcia and Burden, 2012). However these systems do not allow observation and manipulation over long periods of time in live NMJ. Therefore understanding the NMJ's development often needs transgenic organisms and their manipulation involves generation of transgenic organisms which is time consuming and sometimes impossible. To overcome these problems, different \textit{in-vitro} co-culture systems have been set up where motor neuron and skeletal muscle are grown together in order to recapitulate the formation and eventual disruption of the NMJ. To date, co-culture methods established from various species have been described: mouse (Morimoto et al., 2013; Zahavi et al., 2015), rat (Das et al., 2010; Southam et al., 2013), Xenopus (Lu et al., 1996; Peng et al., 2003), chick (Frank and Fischbach, 1979) and also heterologous co-cultures built from motor neuron and muscle cells obtained from different species: rat-human (Askanas et al., 1987), mouse-human (Son et al., 2011), mouse-chick (Soundararajan et al., 2007). However, these co-culture methods resulted in the formation of immature myofibers (thin muscle fiber, with centrally localized nuclei and no transversal triads) with immature sarcomeric structures (Das et al., 2007a; Das et al., 2009; Southam et al., 2013). Moreover, previous models did not take advantage of their co-culture system to analyze other post-synaptic structures like the formation of MuSK and rapsyn clusters which are formed as agrin-induced signaling sparks off and which are essential to the formation of AChR clusters. Here, we describe a new functional co-culture system where muscle fibers from primary murine myoblasts are brought to advanced differentiation and form highly matured NMJs with motor neurons derived from rat spinal cord. The muscle fibers show hallmarks of mature skeletal muscle fiber: peripheral nuclei, transversal triads, myofibrils and organization into three-dimensional bundles performing synchronized contraction. Furthermore the NMJ showed pretzel-like morphology reminiscent
of in vivo synapses. We used this co-culture model to investigate the formation of the post-synaptic apparatus beyond the clustering of acetylcholine receptors (AChRs) and we investigated the role of motor neuron firing on muscle development and differentiation. We found that AChRs form clusters at motor-neuron/muscle contacts and that the post and pre-synapse show hallmarks of maturation and these NMJs are functionally active.
MATERIALS AND METHODS

Animals

All animal experimentation were approved by the Animal Ethics Committee of Pierre et Marie Curie University. E13 pregnant rats were obtained from Janvier Labs, St Quentin Fallavier, France. Primary murine myoblasts were obtained from animals of either sex at postnatal day 7 (P7).

Reagents

Matrigel Basement Membrane Matrix was purchased at Corning Life Sciences (ref 354230). Matrigel protein concentration as obtained by Lowry method ranged between 9.2 and 10.4 mg/ml and endotoxin as measured by Limulus Amoebocyte lysate assay was less than 1.5 EU/ml. Collagenase, recombinant BDNF and insulin were purchased from Sigma (ref C9263, ref B3795, ref I1882 respectively). Dispase II was purchased from Roche (Neutral protease, grade II, ref 04942078001). Chicken Embryo Extract was purchased at MP Biochemicals (MP Biochemicals ref 2850145). Recombinant Rat CNTF and recombinant Rat GDNF were purchased at R&D Systems (ref 557-NT-010, ref 512-GF-010, respectively). Tetrodotoxin (TTX) was purchased at Alomone Labs (ref T-550). Tubocurarine was purchased at Sigma Aldrich (ref T2379).

Primary skeletal muscle culture and differentiation

Skeletal muscle cultures for the aneural system has been previously described (Falcone et al., 2014). Skeletal muscle cultures for the co-culture system were performed as previously described (Falcone et al., 2014) and with the following alterations: Tibialis anterior, extensor digitorum longus, gastrocnemius and quadriceps from thigh muscles were sampled from P7 pups. Muscles were minced manually then digested in collagenase and dispase (0.5mg/ml collagenase and 3.5 mg/ml dispase) at 37°C for 1h30 (Figure 1, day -6). Digestion was stopped with dissection medium consisting of IMDM with Glutamax (Gibco ref 31980-022),
1% penicillin/streptomycin (Gibco ref 15140-122), 10% Fetal calf serum (Eurobio ref CVFFCSF00-01). Cells were centrifuged at 600rpm for 6 minutes and the supernatant was recovered and centrifuged at 1300rpm for 7 minutes. Cell pellet was resuspended in dissection medium and filtered on a 40µm cell strainer (Falcon Corning ref 352340) and resuspended in dissection medium in 100mm petri dishes (Falcon Corning ref 353025) for preplating. Pre-plating was done over 3 hours. 3.5 mm fluorodishes suitable for immunofluorescence (World Precision Instruments ref FD35-100) were coated with Matrigel. After preplating, cells were recovered, centrifuged at 1500 rpm for 10 minutes and resuspended in proliferation medium consisting of IMDM with Glutamax, 1% penicillin/streptomycin 20% Fetal calf serum, 1% chicken embryo extract. Once primary myoblasts have reached 60-70% confluence, cells were switched to differentiation medium consisting of IMDM with Glutamax 1% penicillin/streptomycin 2% Horse serum (Gibco ref 26050088) (Figure 1, day -3). The day after (Figure 1, day -2), myotubes were coated with Matrigel and kept in fresh differentiation medium for 48hrs at 37°C in a 5% CO₂ incubator before plating of spinal cord explants.

**Embryonic spinal cord explants isolation**

Pregnant E13 Sprague-Dawley rats were euthanized with CO₂ (Figure 1, Day 0). Embryo chain was sampled in HBSS (Gibco ref 14170) supplemented with 10% penicillin/Streptomycin. Each embryo was isolated and rinsed in HBSS with Pen/Strep solution and decapitated. Spinal cords were isolated from the rest of the embryo using student vannas spring scissors (Fine Science Tools ref 15001-08/15000-00) and student dumont forceps (Fine Science Tools ref 11200-33/11297-10/11254-20) under binoculars. Blood vessels, muscles and connective tissue were removed delicately so that only the spinal cord with the dorsal root ganglions is left. The spinal cord was cut transversally in small explants with at least one dorsal root ganglion attached to the explant.
**Co-culture of neuronal cells and muscle fibers**

Differentiation medium was removed from muscle myotubes (Figure 1, Day 0). Spinal cord explants were plated very delicately on myotubes (around 4-5 explants per dish) and supplemented with 100µl of co-culture medium consisting of DMEM with glutamax (Gibco ref 61965-026), 25% of medium 199 with Glutamax (Gibco ref 41150-020), 5% FBS, 1% penicillin/streptomycin, 20µg/ml insulin, 10n/ml GDNF, 10ng/ml BDNF, 10ng/ml CNTF. Co-culture medium was added dropwise directly to avoid detachment of the explants. Co-cultures were left at 37°C in 5% CO₂ for 3-4 hours to allow adhesion of explants. Co-culture medium was added very delicately up to 600µl. The following day 400µl of co-culture medium was added to the co-culture. At day 3 post-co-culturing, a layer of Matrigel was used to coat the co-culture in order to provide a three-dimensional environment to the culture and keep structures tightly bound. Medium was changed every two/three days accordingly to cell state. Tetrodotoxin (TTX) was added at day 12 post-co-culturing (final concentration 1µM, Alomone Labs) (Figure1). Co-cultures were fixed at day 14 post-co-culturing.

**Fixation and Immunocytochemistry**

Co-cultures were washed twice with PBS (Gibco ref 14190-094). Fixation was done in either 4% PFA (Electron Microscopy Science ref 15710-S) for 20 minutes at room temperature (RT) or in acetone/methanol solution (ratio 1:1) for 6 minutes at -20°C accordingly to antibodies’ specific requirements. Alpha-bungarotoxin labeling of AChRs was performed with 5µg/ml TRITC-BTX (Sigma Aldrich ref T0195) in PBS for 15 minutes at RT prior to permeabilization and prior to acetone/methanol fixation. After BTX staining, cells were washed twice with PBS. Permeabilization was done in PBS 5% Triton (Sigma Aldrich ref X100) for 5 minutes at RT. Cells were washed twice in PBS and then saturated in BSA 5%, 10% goat serum (Gibco ref 16210-064) for 1hour at RT. Primary antibodies were incubated overnight at 4°C in PBS BSA 5%, saponin 0.1%. Co-cultures were washed 3x 10 minutes
with PBS at RT under slow agitation and stained with corresponding secondary antibodies supplemented with DAPI for 1 hour at RT. Co-cultures were washed 3x 10 minutes with PBS at RT and then mounted in Fluoromount medium (Fluromount-G, Southern Biotech, ref 0100-01) and analyzed using confocal microscopy.

**Primary Antibodies**

The following antibodies were used: mouse anti-βIII tubulin (R&D systems ref MAB1195, clone #TuJ1, 1/400), rabbit anti-Ryanodine receptor (Braubach et al., 2014) (Millipore ref AB9078, 1/200), goat anti-VaChT (Atasoy et al., 2014) (Millipore ref ABN100, 1/100), goat anti-ChAT (Sümbül et al., 2014) (Millipore ref AB144, 1/100), chicken anti-NFH (Wainger et al., 2015) (Millipore ref AB5539, 1/400), mouse anti-DHPR (Bradley et al., 2014) (Abcam ref Ab2864, 1/400), mouse anti-Synaptotagmin (Wong et al., 2014) (Abcam ref ab13259 clone ASV30, 1/100), mouse anti-α-actinin (Falcone et al., 2014) (Sigma Aldrich ref A5044 clone BM-75.2, 1/500), mouse anti-sodium channel pan (Bailey et al., 2003) (Sigma Aldrich clone K58/35 ref S8809, 1/100), mouse anti-Ankyrin (Bailey et al., 2003) (Thermo Scientific ref 33-8800 clone 4G3F8, 1/100), mouse anti-Bassoon (Jing et al., 2013) (Abcam ref ab82958, 1/100), rabbit anti-glial fibrillary acidic protein (GFAP) (Achstätter et al., 1986) (Dako ref Z0334, 1/100), mouse anti-oligodendrocytic marker O4 (Paintlia et al., 2004) (Sigma Aldrich ref O7139, clone O4, 1/400), rabbit anti-MuSK (serum T194, gift obtained from Markus Ruegg, Biozentrum, University of Basel, 1/500), mouse anti-rapsyn (Abcam ref ab11423 (1234), 1/200). Mouse anti-Syne1 (clone 8c3, gift obtained from Glenn Morris, Keele University, 1/200).
Quantification of peripheral nuclei

Myofibers were stained for DHPR and DAPI and acquired with a Leica SPE confocal using a 40x 1.15 NA ACS Apo objective at different z positions. Nuclei extruding the myofiber periphery were considered as peripheral. A minimum of 20 fibers were counted per condition in three independent experiments.

Quantification of transversal triads

Myofibers were stained for DHPR and DAPI and acquired with a Leica SPE confocal 40x 1.15 NA ACS Apo objective. Fibers with more than 50% transversal triads were scored as positive. A minimum of 20 fibers were counted per condition in three independent experiments.

Fiber thickness quantification

Myofibers were stained for RyR and acquired with a Leica SPE confocal 40x 1.15 NA ACS Apo objective. Average of three measurements per fiber was calculated for fiber thickness. A minimum of 20 fibers were counted per condition in three independent experiments.

Fluorescence and Live Imaging

Epi-fluorescence images were acquired using a Nikon Timicroscope equipped with a CoolSNAP HQ2 camera (Roper Scientific), an XY-motorized stage (Nikon), driven by Metamorph (Molecular Devices) and 4x 0.13NA, 10x 0.30NA and 20Xx 0.45NAPlanApo oil immersion objectives. Confocal images were acquired using Leica SPE confocal microscope with a 40x 1.15 NA ACS Apo objective. Live imaging was performed in an insulated temperature controlled chamber (Okolab) to maintain cultures at 37°C and 5% CO₂ (Okolab) using a 20x0.3 NA PL Fluo dry objective.
**Image analysis**

Images were analyzed using Image J software (imagej.nih.gov/ij/). Images of z-projections are specified in corresponding figure legends. 3D rendering (Figure 5b') was performed using Voxx 2.1 software (Indiana University).

**Tetrodotoxin experiment**

For action potential blocking experiments, the selective Na\(_V\) channels blocker, Tetrodotoxin (final concentration 1µM, Alomone Labs) was applied to the co-culture medium. Response in myofibers was recorded by video microscopy at 2fps and analyzed using Metamorph 7.1 (Molecular Devices). Contractions of myofibers were monitored by following displacement of the plasma membrane of one point over time using the Track points plug in.

**Fluorescence in situ hybridization**

For RNA FISH, we modified the protocol provided by Stellaris (Biosearch Technologies CA, USA). Briefly, cells were first incubated with Bungarotoxin (5µg/ml) conjugated with a 488nm fluorophore and washed twice with PBS prior to fixation with 4% formaldehyde and permeabilization with 70% ethanol for 10 min at 4°C. Probes conjugated with 570nm fluorophore against AChR\(\varepsilon\) (Stellaris) were incubated at 125nM in 100 mg/mL dextran sulfate and 10% formamide in 2X SSC with cells for 16h at 37°C. After wash with 10% formamide in 2X SSC, cells were incubated with primary antibody against Tuj1, followed by incubation with secondary antibody and DAPI at 5ng/ml. After washes with 10% formamide in 2X SSC, cells were covered by Vectashield and directly imaged using a widefield fluorescent microscope.

**Electrophysiology recordings**

For technical purposes, electrophysiological studies were performed in conditions of diluted Matrigel. These changes lead to decrease myofiber differentiation, but we postulated that if in these conditions, the NMJs were functional, it would allow to demonstrate functionality in
the usual co-culture conditions. Intracellular recordings were performed at room temperature in a solution containing (in mM): 145 NaCl, 3 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES (pH 7.4) and 11 glucose. Sharp pipettes were made from borosilicate glass (Clark Electromedical Instruments Reading, England), pulled on a P-1000 puller (Sutter Instrument Company, Novato, CA, U.S.A.) and had a resistance of 40-60 MΩ when filled with a 3M KCl solution. Membrane potential was recorded using a SEC 0.5X amplifier (NPI electronic GmbH, Tamm, Germany) and digitized by a 16 bit A/D converter (Digidata 1322A, Axon Instruments, Union City, CA, U.S.A.).

**Statistical analysis**

Statistical significance was determined using GraphPad Prism (GraphPad Prism Software Inc. version 6). Statistical tests used have been mentioned in the corresponding figure legends. P≤0.05 was considered as significant.
RESULTS

Development of a heterologous co-culture system

We have previously described a method to obtain highly differentiated myofibers in vitro, potentially useful to study myoblast fusion, nuclear movement, myofiber differentiation and formation of agrin-induced AChR clusters (Falcone et al., 2014). However, this method is not suitable to study formation of NMJ and the post-synaptic apparatus as well as mechanisms of denervation-dependent muscle atrophy, due to the lack of two of the basic cell types forming the NMJ: neurons and Schwann cells. We therefore developed an easy co-culture system which allowed us to obtain highly differentiated myofibers in a more physiological context, i.e. innervated by neurons.

Myoblasts were isolated from P7 mouse pups and plated on Matrigel coated coverslips (Day -6, Figure 1A). Matrigel is rich in extracellular matrix proteins important for muscle differentiation (Falcone et al., 2014). Moreover Matrigel represents a very suitable biomaterial for our co-culture given the presence of laminin in its components (Kleinman et al., 1982), an important element in presynaptic differentiation and organization of NMJ active zones (Nishimune, 2012; Sanes and Hall, 1979). In addition, Matrigel provided a three-dimensional matrix for our cells to grow and differentiate. Myofibers were then switched to differentiation (Day -3, Figure 1). 24 hours later (Day -2, Figure 1), another layer of Matrigel was added to the cells to cover the differentiating muscle fibers (Figure1). 48 hours later (Day 0, Figure 1), whole transverse sections of the spinal cord with attached dorsal root ganglion (DRGs) without removal of the ventral part, i.e. the ventral horn, where efferent nerves are believed to emanate (Blits et al., 2004), were plated on myotubes (Figure 1). Medium was also supplemented with growth factors (BDNF, CNTF and GDNF) involved in the maturation of NMJs (Peng et al., 2003; Sakuma and Yamaguchi, 2011; Tuttle and Matthew, 1995; Zahavi et al., 2015). Therefore we used sections with the DRGs. After 12 days, myofibers showed high contractile activity and to reduce the detachment of the neuron-
muscle structures from the dish, we used Tetrodotoxin 1µM (TTX). This treatment, not only reduced myofiber contractions, but also enhanced formation of neuromuscular junctions. Time course analysis showed that at 14 days after starting the co-culture, fully mature myofibers and functional NMJs were present. In addition, we found that the co-culture could be kept over longer time periods (at least 30 days) without affecting the viability of the neurons or myofibers, suggesting that we were able to generate a system stabilized by a complex dialogue between different cell types.

**Characterization of co-culture morphology**

Study of the morphology of the co-cultures over time showed that at Day 1 post-culturing, the explants already projected nerve processes (Figure 2A). If, by Day 1, explants have not shown any nerve processes, the explants will certainly not adhere to the muscle and were removed. At Day 3, neuron-muscle contacts could already be spotted in the cultures (Figure 2B, b'). At Day 5, the nerve processes have extended very widely (Figure 2C) and cell types other than neurons were present in the cultures (Figure 2C) but muscle fibers still show immature differentiation status (Figure 2D) and no alignment as shown by the colored outlines of myofibers (Figure 2d'). At Day 13, however, myofibers showed hallmarks of differentiation such as peripheral nuclei (Figure 2F, 2G red arrows) as well as numerous contacts with neurons (Figure 2G, g'). Movement of myonuclei to the periphery, though not completely understood yet, has been described as an important characteristic of muscle differentiation and failure of re-positioning of myonuclei from center to periphery is pervasive in centromyonuclear myopathy like X-linked myotubular myopathy (Folker and Baylies, 2013). While at Day 5, muscle cells showed random positioning in the culture, interestingly at Day 13, the muscle fibers showed alignment around the explant(Figure 2H, 2I) evocative of the formation of bundling of multiple myofibers *in vivo*. In Figure 2i',
outlines of myofibers allow to distinguish the regular myofiber alignment. Altogether, these results showed that our protocol allowed for the formation of axonal processes, muscle-nerve contacts and features of muscle differentiation over a short time period.

**Characterization of co-culture cell components-neuronal populations**

We investigated the presence of different cell types by first looking at the presence of cholinergic motor neurons, known to form the NMJ upon contact with the muscle (Feng and Dai, 1990). To this end, we marked the co-cultures with vesicular acetylcholine transporter and choline acetyltransferase (VaChT and ChAT) antibodies (Schäfer et al., 1998) and found that the co-cultures contain many cholinergic neurons (Figure 3A, B).

The presence of cells, other than neuron and muscle has not been previously described in methods of co-cultures. We were much interested in the presence of glial cells, especially Schwann given their importance in the formation and nurturing of the NMJ (Sanes and Lichtman, 1999; Witzemann, 2006). To this end we used the following macroglial cell markers: glial fibrillary acidic protein (GFAP) which marks non-myelinating Schwann cells (Jessen and Mirsky, 2005; Jessen et al., 1990; Kegler et al., 2015) and the oligodendrocytic marker O4 which marks oligodendrocytes (Gorris et al., 2015). Schwann cells are indispensable for nerve capping and production of the myelin sheath which speeds conduction of the action potential along axons and insulates the latter to avoid energy loss (Jessen and Mirsky, 2005; Sanes and Lichtman, 1999). Oligodendrocytes are the alter-ego Schwann cells in central nervous system (CNS). We found both Schwann cells and oligodendrocytes, the latter probably emanating from the ventral horn (Wada et al., 2000), in our co-cultures, at Day 14 (Figure 3C and Figure 3D). Interestingly, both cells types showed contact points with neurons suggesting capping of axons like described *in vivo* (Figure 3D inset, Figure 3D inset). Overall, the presence of these different neuronal cell types, with a
localization resembling what is known in vivo, supports the notion that our system enables the development of efficient motor neurons.

**Characterization of co-culture cell components-myofibers**

We previously described an in vitro model of differentiated myofibers displaying several features indicative of maturation, among which the presence of T-tubules and sarcoplasmic reticulum (SR) evenly and transversally organized (Falcone et al., 2014). These differentiated myofibers were formed in the absence of neurons, and therefore we named it aneural system. To characterize the maturation of myofibers in our co-culture and aneural systems, we used antibodies against the dihydropyridine receptor (DHPR), a voltage-gated channel, found at the T-tubule or against Ryanodine receptor (RyR), found at the sarcoplasmic reticulum membrane (Flucher et al., 1993; Flucher et al., 1994). Both receptors are implicated in excitation-contraction (EC) coupling mechanism, through the existence of triads, where one T-tubule is coupled to two terminal cisternae of the sarcoplasmic reticulum. At Day 14, myofibers in the co-culture system showed features of high differentiation: well formed DHPR positive triads and peripheral nuclei (Figure 4A, nuclei: arrows). In the co-culture system, we found that the percentage of myofibers with peripheral nuclei was similar in both systems (Figure 4B). However, the number of fibers with triads was higher than at the endpoint of the aneural system (Day 10) (mean ± sem 78.6% ±2.3 vs 47%±5.6, Figure 4C).

To further characterize the degree of muscle differentiation, we stained our co-cultures for α-actinin, an actin-binding protein found at the z-disk. α-actinin binds actin and several other proteins like titin and forms a lattice-like structure, important for the stabilization of the muscle contractile apparatus (Sjöblom et al., 2008). Day 14 myofibers showed regular alternation between the z-disk and doublets structure marked with α -actinin and RyR respectively as shown on the line scan (Figure 4D and d', RyR doublets are marked with arrows).
Comparison between myofiber thickness from the co-culture and aneural system confirmed that the presence of neurons definitely promoted the formation of thicker fibers (Figure 4E). Myofibers from the co-culture showed a 1.8 fold increase in thickness (mean ± sem 17.4µm±0.72vs 9.38±0.36, Figure 4F). Of note, when Matrigel was substituted with laminin, a component of muscle basal lamina, the differentiation status of myofibers was negatively affected suggesting that laminin alone, though intensively nourishing to myofibers, could not account for high myofiber differentiation (Figure 4G). Furthermore, as we described in Figure 2H, myofibers showed bundling like *in-vivo* fasiculus, an important condition to increase contractile strength (Figure 4H). Taken together, these data demonstrate that our protocol allows for robust differentiation of thick myofibers with peripheral nuclei and transversal triads and importantly that the presence of neurons is a determining factor in the differentiation process of myofibers.

**Formation of NMJs**

NMJ are characterized on the postsynaptic membrane (the myofiber plasma membrane) by the presence of highly-clustered acetylcholine receptors, detected by α-bungarotoxin (Sanes and Lichtman, 1999; Wu et al., 2010). In several previous protocols which described NMJ formation, the researchers have presented NMJs as random colocalization points between the neuron and the myofiber with dotty and/or unstructured AChR clusters (Das et al., 2007b; Southam et al., 2013), usually formed before any innervation. However, *in vivo*, neuron-muscle fortuitous colocalization alone is not a satisfactory feature (Thomson et al., 2012).

Early characterization of NMJ has shown that, as opposed to small unspecialized aneural prepatterned AChR clusters, axons end perfectly at the post-synaptic terminal and overlap the latter where AChR clusters will mature to form complex structures (England and Rubin, 1987; Sanes and Lichtman, 1999). Remarkably, in our co-culture system at Day 14, axons extended to innervate myofibers with underneath complex AChR clusters with
similar morphology of NMJ found in vivo, with the characteristic pretzel shape with typical internal perforations, an indication of low density AChR spots (Figure 5A). We also found few unstructured AChR clusters at un-innervated myofibers (Supp Figure 2). Given that three layers of Matrigel were used in order to create an elaborate 3D matrix, we analyzed orthoslices of NMJ stacks and found elaborate capping of AChR clusters by nerve terminals with presence of underneath synaptic nuclei. Complexity of NMJs structures (Figure 5B) was revealed using 3D reconstruction where myofiber channel was removed to take full account of the AChR clusters capping by the neuron (Figure 5b’ and inset).

The NMJ is also characterized by the accumulation in the myofiber of specific mRNAs such as the AChR epsilon subunit mRNA (Gu and Hall, 1988; Merlie and Sanes, 1985) and subsynaptic nuclei accumulate Syne-1 protein at the nuclear envelope (Apel et al. 2000). We found that AChR epsilon subunit mRNA accumulates at the NMJ in our co-culture system (Figure 5C). Furthermore, subsynaptic nuclei also accumulate more Syne-1 at the nuclear envelope (Figure 5D). Additionally, we further investigated whether the NMJ bore active zones at presynaptic terminals. To this end, we stained for Synapsin I, a synaptic vesicle protein, synaptotagmin, a calcium sensor also important for vesicle docking process and Bassoon, a scaffolding protein believed to guide the synaptic vesicles to the active zones (Fornasiero et al., 2012; Reist et al., 1998; Südhof, 2012; Willig et al., 2006; Zhai et al., 2001; Ziv and Garner, 2004). We found that all the proteins were enriched specifically at the presynaptic terminals of the NMJ. (Figure 5E, F, Supplementary Figure 3).

Finally, we found that the growth factors that were supplemented at Day 0 (BDNF, CNTF and GDNF) were crucial for the formation of the NMJ since the number of NMJs was dramatically reduced in the absence of the growth factors (mean ± sem, 10±1 vs 2±1, Figure 5G). We tested co-cultures with transverse section of ventral root stripped of meninges and DRGs in same condition with growth factors and found, in accordance with other reports
that the number of NMJs was drastically reduced (Guettier-Sigrist et al., 1998; Kobayashi et al., 1987) (Figure 5H).

Following our data showing that our co-culture system leads to the formation of highly differentiated NMJs, we sought to assess whether our system could allow us to study specifically the regionalisation of different post-synaptic elements. To this end, we investigated the spatial organization of different components, Muscle specific tyrosine kinase (MuSK), found at the membrane of primary gutters like AChR and Rapsyn essential for the formation and maintenance of the NMJ (Wu et al., 2010). Upon neuronal agrin secretion, several signaling pathways are activated at the post-synapse (Luo et al., 2003). Among these pathways is the dimerization and self-activation of MuSK via phosphorylation (Hopf and Hoch, 1998; Kim et al., 2008; Zhang et al., 2008). Subsequently, interactions between the kinase and other synaptic proteins will increase and trigger postsynaptic differentiation. These events are crucial to AChR clustering. Agrin also induces association of AChRs with Rapsyn, an important stabilizer of AChR clusters (Apel et al., 1997; Chen et al., 2007). We found that MuSK and Rapsyn colocalize with AChR clusters AChRs as expected (Figure 6A, B). Interestingly, MuSK could be found at the muscle plasma membrane, in line, with its receptor tyrosine kinase functions (Figure 6A). We also investigated the localization of ankyrin G, known to be spatially segregated from AChR at the NMJ in early development and then found at secondary junctional folds later in development (Trinidad et al., 2000; Wood and Slater, 1998). We found that ankyrin G was clustered in close proximity with the NMJ and found at the muscle membrane but not completely at the NMJ (Figure 6C) suggesting that at this time of development of the system, the protein localization has not reached a fully mature status as previously reported in vivo (Bailey et al., 2003), further supporting this method for the study of different phases of NMJ maturation.
Overall, these data demonstrate the uniqueness and robustness of our system to study NMJ formation and post-synaptic development.

**NMJs functionality**

The structural characterization of our co-culture showed the presence of different basal elements organized specifically to allow for a functional neuromuscular system, from the motor command to the myofiber contraction. Both innervated myofibers and aneural myofibers showed contractions (Figure 7A, Additional File 2. Figure 2A, Additional File 4. Video 1 and Figure 7A, Additional File 2. Figure 2A, Additional File 5. Video 2). The contractile activity by itself showed the functionality of the contractile apparatus and of the excitation-contraction coupling. Indeed, 1 µM Tetrodotoxin (TTX), a selective blocker of the voltage-gated sodium channels, abrogated contractile activity in both neural (Figure 7A, Additional File 2. Figure 2B and Additional File 4. Video 1) and aneural cultures (Figure 7A, Additional File 2. Figure 2C and Additional File 5. Video 2). This suggests that sodium action-potentials activate normally the DHPR voltage-sensors at the level of the T tubules, and subsequently the calcium release from the terminal cisternae of the sarcoplasmic reticulum and the contraction. At first glance, the contractile activity however does not allow to conclude on the efficiency of the synaptic transmission, since myofibers in aneural culture spontaneously twitch (Figure 7A, Additional File 2. Figure 2A, Additional File 5. Video 2). Nonetheless, a more detailed analysis showed that the temporal pattern of the contractile activity changed in the presence of explants after putative NMJs are formed. While aneural cultures exhibited rhythmic twitching activity in some individual myofibers, contractile activity in co-cultures, at proximity of the explants, was arrhythmic, with longer contraction events, and was observed in a higher number of myofibers than in aneural cultures (Figure 7A, Additional File 2. Figure 2A, Additional File 4. Video 1). In addition, myofibers which have bundled together tend to contract simultaneously, as it would have been the case in a
motor unit, i.e. a set of myofibers innervated by the same motor neuron. Electrical activity being known to participate to the myofiber development (Dutton et al., 1993) we tested the effect of TTX on muscle differentiation. When TTX was applied early to the co-culture (Day 4 to Day 14), differentiation of myofibers was severely affected and resulted into decreased number of peripheral nuclei (mean ± sem, 82.5%±5.5 vs 49%±13, Figure 7B) and transversal triads (mean ± sem, 67.5%±4.5vs 7%±7, Figure 7C).

In order to test whether synaptic transmission is functional and responsible for the arrhythmic activity in our co-culture we performed electrophysiological recordings. Electrophysiological studies were performed in conditions of diluted Matrigel. These changes lead to decrease myofiber differentiation, but we postulated that if in these conditions, the NMJs were functional, it would demonstrate functionality in the optimum co-culture conditions. In twitching myofibers, membrane potential recordings showed the regular muscle firing already described in aneural mice cultures (Sciancalepore et al., 2005) (Figure 8A). This spontaneous firing is independent of synaptic activity, and is rather due to the activation of a T-type calcium current in the sub-threshold range of the membrane potential, which raises the membrane potential to the action-potential threshold (Figure 8A inset). This regular firing, as well as its associated contraction activity, was insensitive to 50 µM curare, an antagonist of the nicotinic receptors. In irregular- contracting myofibers, the electrical activity was made of non-rhythmic sub-threshold depolarizations and action-potentials (Figure 8B). Figure 8C shows examples of these transient depolarizations, in absence of spikes, showing their sub-threshold amplitudes or depolarization-induced inactivation of the sodium channels. Their individual temporal pattern perfectly fitted those of nicotinic post-synaptic potentials (PSPs) as usually recorded in adult murine innervated muscles, strongly suggesting that the synaptic transmission is functional in the co-cultures (Ouanounou et al., 2016). The nicotinic nature of these PSPs could finally be confirmed by blockade with curare,
a specific nicotinic receptors antagonist (Figure 8D). Curare was puffed in the recording chamber (50 µM final concentration) and the diffusion time allowed to observe the gradual blockade of the post-synaptic potentials (Figure 8D, insets). Supra-threshold post-synaptic potentials were responsible for the irregular firing in these innervated myofibers, and were visible at enlarged time scale in the milliseconds preceding the spikes (Figure 8B, insets). Figure 8E shows a recording in an innervated myofiber exhibiting both spontaneous and synaptic-induced activity. In conclusion, though the conditions had to be modified for electrophysiological recordings, the data demonstrated the functionality of the nicotinic synaptic transmission in the co-culture.

Altogether these results provide evidence for the formation of functional NMJs between neurons and highly differentiated myofibers.
DISCUSSION

Here we described a heterologous co-culture system capable of inducing sturdy differentiated NMJs and myofibers \textit{in vitro} that can be directly observed by time-lapse microscopy over the whole period of differentiation. Furthermore, the myofibers can be manipulated by transfection with siRNAs and shRNAs to downregulate protein levels, or by overexpression of plasmids encoding multiple proteins (Falcone et al., 2014). To our knowledge, such high differentiation stage of NMJ have never been obtained in \textit{in vitro} conditions, therefore this system offers unique conditions for the study of the complex mechanisms involving the formation of NMJ and differentiation of myofibers, which are difficult to identify using the current available \textit{in vivo} systems.

This method is very practical to implement in terms of availability and abundance of material and is time-saving. One pregnant rat usually bears a mean of 13-15 embryos, providing an important amount of material to get spinal cord explants, allowing for the assessment of multiple culture conditions in parallel. In the present study, functional and highly differentiated NMJs, neurons and myofibers were obtained already at 14 days (Figure 5). Other studies had used lengthy protocols where formation of NMJs, was obtained after 20-25 days or even longer time periods, if we take into account the time needed for either prior MN differentiation or myofiber differentiation (Das et al., 2010; Southam et al., 2013). In addition, in these studies, the NMJs and myofibers did not reach the same stage of differentiation as the one described in this manuscript.

We used spinal cord explants with dorsal root ganglion (DRG) as a source of neuronal populations (Askanas et al., 1987; Zuchero, 2014). In several studies, however, the spinal explant was stripped of DRGs prior to plating with muscle cells to enrich the culture in motor neurons. The reason being that the dorsal root ganglion give rise to afferent sensory neurons, which are supposed to vehicle the electrical impulse from the peripheral nerve system to the
brain, rather than efferent α-motoneurons, which triggers muscle contraction (Southam et al., 2013; Zahavi et al., 2015). We tried to co-culture rat spinal cord's ventral horn (stripped from DRG) in the same conditions as described in Figure 1. Surprisingly the number of NMJs was smaller than the one we could reach in our present setting. This prompts the consideration that specific neuronal populations arising from the dorsal root, among which afferent neurons, could possibly be important for the full functioning of the system as suggested in-vivo (Zhang et al., 2015). Indeed, these neurons could signal, in turn, differential contractile status of the muscle to the α-motoneurons. This also suggests that the complete spinal cord transverse section (with the DRGs) probably host progenitors cells necessary to differentiate into possible NMJ's feeder and sustaining cells.

Our system has the unique capacity of forming highly differentiated myofibers harboring highly differentiated endplates. However, our keen interest was to characterize differentiation of muscle fibers in a neuronal context, which takes after in-vivo conditions. Much attention has been given to the development and improvement of the neuronal compartment with the emergence of embryonic stem cells (Chipman et al., 2014; Das et al., 2007a; Soundararajan et al., 2007) and induced pluripotent stem cells (Bohl et al., 2015; Burkhardt et al., 2013; Egawa et al., 2012), however, the muscle counterpart has been underexploited and its potential underestimated. Previous studies failed to produce convincing data on muscle differentiation either because muscle cell line, like C2C12, incapable of producing highly differentiated myofibers were used or because the muscle compartment was overlooked (Arnold et al., 2012; Chipman et al., 2014; Das et al., 2009; Das et al., 2010; Umbach et al., 2012). The use of such cell lines might as well preclude synaptogenesis and/or synaptic differentiation. Here we achieved substantial differentiation of myofibers which showed peripheral nuclei, well organized transversal triads, and thickness to a higher extent to what we previously reported in our aneural myofiber cultures (Falcone et
al., 2014) (Figure 4). This method provides, therefore, a relatively high developmental environment both mechanically (via neuronal impulses) and chemically, given the multiple growth factors in the Matrigel, for muscle growth and maturation (Maffioletti et al., 2015). The effect of Matrigel constituents on muscle progenitor populations and hence muscle growth and differentiation has already been proven (Grefte et al., 2012) and could explain why our system results in thicker and more differentiated myofibers in comparison to other protocols not using Matrigel. Among the different Matrigel constituents, the insulin growth factor-1 (IGF-1) is known to enhance DHPR function and expression and henceforth EC machinery, important to convert chemical signaling into mechanical signaling which is correlated to muscle development and thickness.

This system also allows long-term survival of the components. Co-cultures could be kept more than 30 days with muscle fibers still contracting. It is probable that such co-cultures could be kept for even longer time periods as it has been shown previously with dorsal root ganglion spinal cord explants and primary myoblasts co-culture (Askanas et al., 1987). The longevity of the culture would be advantageous to study motor neuron diseases which encompass slow deterioration of the NMJ. As such, subcellular post-synaptic changes could be followed over time, for instance, with live imaging.

With the advances made in the field of embryonic stem cells (ES) and induced pluripotent cells (iPSc), we think that our system can be useful to understand the basic condition necessary to synaptogenesis. In fact, although, any motor neuron could form a NMJ with any muscle fiber, additional signals will favor the NMJ formation in favor of the ideal partners. This can only be achieved in the presence of all required cell types. We believe that although new models derived from ES-MNs and iPSC-MNs allow for homologous co-cultures systems and also hold promise for personal-targeted therapies, these systems might lack some of the required cues that allow for the precise formation of the neuromuscular
junction structures like junctional folds in the muscle fiber (Chipman et al., 2014; Son et al., 2011). Moreover, maintenance of the co-culture over time in these systems is fragile and still needs to be solved. The issue of compromised life-time in stem cell cultures mainly arises from the differences in cell culture media requirements. ES-derived human motor neurons are infamously difficult to culture and can develop only in complex culture medium requiring diverse trophic factors as supplements. Over time, these culture media can be pernicious to skeletal muscle. Although, these techniques should be given all due interest, the cost, time and technical skills involved are not insignificant. Here we provide an interesting alternative technique which is more time- and cost-effective.

Because one of the major technical breakthroughs in NMJ formation study has been the microfluidics system allowing to separate spatially the neuronal component from the muscle component, the described system can be adapted for the use with microfluidic devices. We believe that such a method could allow both (i) to grow all necessary neuronal cells (neurons and glial cells) and (ii) the natural dynamics of paracrine secretion of trophic factors important for synaptogenesis to occur, like suggested in the recent work of Zahavi et al (Zahavi et al., 2015).

To summarize, our work can be a valuable tool to study the formation and development of the NMJ. We believe that because the NMJ is a perfect spatial and chemical organization between neurons, muscle and glial cells, studying all these components together could permit better understanding of the physiology of NMJs and the temporal defaults occurring in motor neuron diseases like ALS, spinal bulbar palsy and SMA.
CONCLUSION

Our objective was to set up a new easy and durable co-culture system that would enable the fast and robust differentiation of myofibers and the formation of functional NMJs. We made use of Matrigel to create a culture platform which allowed 3D growth of neuron-muscle structures. Our method achieved formation myofibers exhibiting features of high differentiation as well as formation of functional NMJs with post-synaptic specialization. This model can be used for pharmacokinetics and drug design purposes in NMJ-defects associated pathologies like MNDs.
Abbreviations: NMJ: neuromuscular junction, DHPR: dihydropyridine receptor, RyR: Ryanodine receptor

Conflict of interest:
The authors declare no conflict of interest

Author’s contributions:
VV, BC and ERG conceived and designed the project. VV performed and analyzed all the experiments and was assisted by BC for Figures 5B, 7 and supplementary Figure 2. BC performed the FISH experiments. GO performed electrophysiology. VV wrote the manuscript and BC and ERG revised and edited it. All authors read and approved the final manuscript.

Acknowledgments:
We greatly acknowledge Anne-Sophie Arnold for experimental instructions. We thank Sestina Falcone and Vanessa Ribes for reagents and help with mouse dissection. We thank Sonia Alonso-Martin and Bernadette Drayton for reagents. We also thank Stéphane Nédelec and Gomes lab members for helpful discussion. We thank Alain Schmitt for technical instructions. We are grateful to Markus Rüegg for the MuSK antibody and Glenn Moris for the Syne-1 antibody. This work was supported by an Agence Nationale de la Recherche (ERA-NET-E-RARE 2) grant no J13A170, by the European Research Council and EMBO installation to ERG.
REFERENCES


Figure 1. Timeline for spinal cord explant and murine myofibers co-culture. Days are expressed relative to day of spinal cord explant plating on myotubes. Fixation of co-culture was done at Day 14 for staining purposes but co-cultures may be maintained for up to 4 weeks with regular medium changes.
Figure 2. Morphological characterization of co-culture.
A) Day 1. Spinal cord explant showing extensions of nerve processes (arrows) on top of primary myoblasts.
B) Day 3. Nerve processes (arrows) forming contact with myotubes (arrowheads), magnified in b’.
C) Day 5. Nerve processes are longer and extended over large distances away from the spinal cord explant.
D) Day 5. Myotubes are still immature. They do not show peripheral nuclei (see magnification). As shown by colored lines highlighting outlines of myofibers in d’, the latter do not show alignment.
E) Day 13. Myofibers with peripheral nuclei, a hallmark of differentiation (red arrows).
F) Day 13. Myofibers show peripheral nuclei (red arrows) and multiple contacts with nerve processes (arrows) magnified in f’.
G) Day 13. Myotubes form bundles around the spinal cord explant. Metamorph software was used to acquire adjacent images with a 4x objective so as to cover a surface of 1.5cm in height and 1cm in width.
H) Day 13. Myofibers show bundling and their regular alignment is shown in h’.
A-G) scale bars, 100µm.
Figure 3. Characterization of neuronal populations at Day 14.
A) Representative images of co-culture stained for VaChT (green) and DAPI (magenta).
B) Representative image of co-culture stained for ChAT (green) and DAPI (magenta).
C) Representative image of co-culture stained for β-III Tubulin (TuJ1) (green), GFAP (red) and DAPI (blue).
D) Representative image of co-culture stained for β-III Tubulin (TuJ1) (green), O4 (red) and DAPI (blue).
A-D) Scale bar, 20µm
Figure 4. Characterization of myofibers at Day 14.
A) Representative z-projection of differentiated myofiber stained for DHPR (green), RyR (red) and DAPI.
B) Quantification of myofibers with peripheral nuclei in aneural vs neural conditions. Error bars, s.e.m., 67 myofibers in aneural and 87 myofibers in co-culture have been counted in n = 3 independent experiments. P-values from Welch-test.
C) Quantification of myofibers with transversal triads in aneural vs neural conditions. Error bars, s.e.m., 67 myofibers in aneural and 87 myofibers in co-culture have been counted in n = 3 independent experiments n = 3. P-values from Welch-test.

D) Representative z-projection of differentiated myofiber stained for RyR (green), α-actinin (red) and DAPI. In d', line scan of boxed region in D) showing average intensity of RyR compared to α-actinin. Arrowheads indicate doublets.

E) Representative images of differentiated myofibers in aneural (up) and co-culture (down) conditions.

F) Quantification of myofiber thickness in aneural vs co-culture conditions. Error bars, s.e.m., 44 myofibers in aneural and 40 myofibers in co-culture have been counted in n = 3 independent experiments. P-values from Mann-Whitney-test.

G) Representative image of myofibers, stained for DHPR (grey) and DAPI, grown on Matrigel vs laminin in aneural vs neural conditions.

H) Representative z-projection image of myofiber bundle stained for RyR (green) and dystrophin (red) in co-culture conditions.

A, D-H) scale bar 20µm.
Figure 5. Characterization of neuromuscular junction at Day 14.
A) Representative image of co-culture stained for TuJ1 (green), AchRs (α-BTX) (red), RyR (grey) and DAPI. Colocalization of AchR clusters with nerve terminal is magnified.
B) Representative z-projection of a co-culture showing NMJ complexity. Orthogonal view in x-axis and y-axis confirm colocalization of AchR nerve endings. b’) 3D reconstruction image of neuromuscular junction in B without myofiber, showing interaction between AChR clusters and nerve endings.
C) In situ hybridization detects AChR epsilon at NMJ. Presence of AChR epsilon is detected with a red fluorescent probe (marked in green in the figure), presynaptic terminal stained for TuJ1 (gray), post-synaptic terminal for α-BTX (red) and DAPI.
D) Representative image of presynaptic terminal stained for NFH (green), α-BTX (red), Syn-1(white). Extrasynaptic nuclei of same fiber indicated with an arrow show decreased Syn-1 expression compared to synaptic nucleus indicated with arrowhead.
E) Representative image of co-culture stained for TuJ1(green), α-BTX (red), Synapsin I (gray).
F) Representative image of presynaptic terminal stained for TuJ1 (green), α-BTX (red) synaptotagmin (gray).
G) Quantification of NMJs with or without addition of BDNF, GDNF and CNTF. Error bars, s.e.m., n = 3 independent experiments. P-values from Welch-test.
H) Quantification of NMJs in condition of whole spinal cord explants vs ventral root explants. Error bars, s.e.m, n=3 independent experiments. P-values from Welch-test.
A, E) scale bar, 10µm
B-F) scale bar, 20µm.
Figure 6. Characterization of post-synaptic specialization at Day 14.
A) Representative z-projection of differentiated myofibers stained for α-BTX (red), TUJ1/MuSK (green).
B) Representative z-projection of differentiated myofibers stained for α-BTX (red), NFH/Rapsyn (green).
C) Representative image of differentiated myofibers stained for α-BTX (red), NFH/Ankyrin G (green).
A-C) scale bar, 20µm.
Figure 7. Differences between muscle contraction in aneural vs co-culture conditions.
A) Average amplitude of contraction observed over the 80 seconds (equal to 160 frames) has been calculated for each condition (+/- TTX). Error bars, s.e.m, n=160.
B) Quantification of myofibers with peripheral nuclei with or without addition of TTX at early time points (day 4 until day 14). Error bars, s.e.m., n = 3 independent experiments. P-values from Welch-test.
C) Quantification of myofibers with triads with or without addition of TTX at early time points (day 4 until day 14). Error bars, s.e.m., n = 3 independent experiments. P-values from Welch-test.
**Figure 8. Intracellular recordings of the myofibers membrane potential.**

A) Lower trace: representative recording of the spontaneous electrical activity found in some myofibers, independently of innervation. Upper trace: enlarged time scale showing the instants preceding spike. Depolarization to the spike threshold is due to the activation of a T-type calcium current (Sciancalepore et al., 2005).

B) Lower trace: membrane potential recording in an innervated and non-spontaneously active myofiber. Arrows mark the occurrence of two sub-threshold postsynaptic potentials (PSP). Upper trace: enlarged time scale showing the instants preceding the spike and pointing out the PSP triggering the spike.

C) PSPs in two different innervated myofibers (left and right traces respectively).

D) PSPs blockade with 50µM curare puffed in the recording chamber. Upper traces show the individual PSPs before and after curare application. Sensitivity to curare confirmed the nicotinic nature of these PSPs.

E) Example of recording showing a combination of spontaneous firing with synaptic-induced spikes. Arrows mark the spikes induced by synaptic events. Right traces: enlarged time scale for two spikes induced by PSPs.