Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process

Laura Nadal Magriñà

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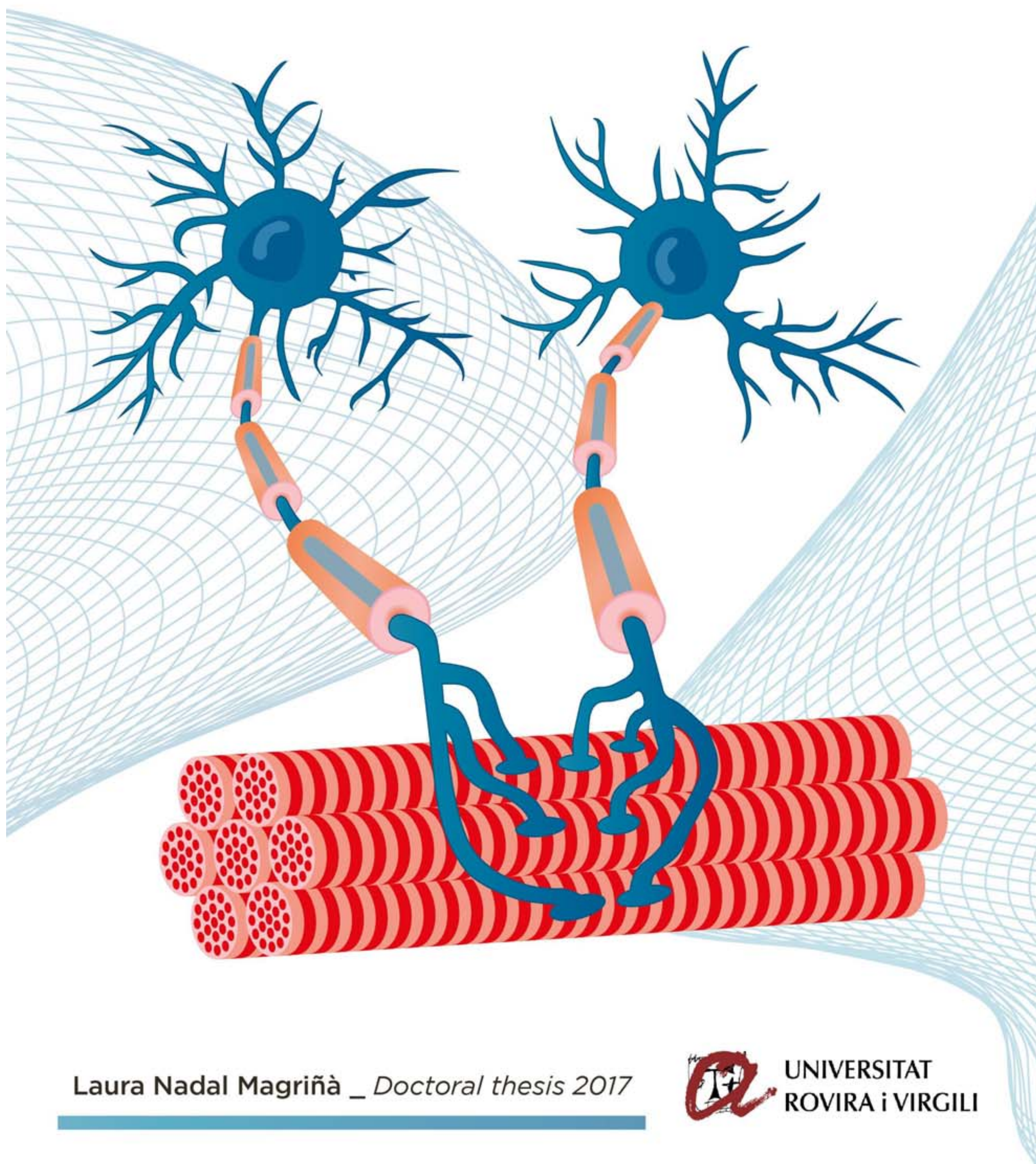

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Muscarinic, adenosine and tropomyosin-related kinase B receptors modulate the neuromuscular developmental synapse elimination process

DOCTORAL THESIS

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FAIG CONSTAR que aquest treball, titulat '*Muscarinic, adenosine and tropomyosin-related kinase B receptors modulate the neuromuscular developmental synapse elimination process*', que presenta Laura Nadal Magriñà per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Ciències Mèdiques Bàsiques d'aquesta Universitat i que acompleix els requeriments per poder optar a Menció Internacional.

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning".

Albert Einstein

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iii. ABBREVIATIONS

A_1	Adenosine A_1 receptor
A_{2A}	Adenosine A_{2A} receptor
A_{2B}	Adenosine A_{2B} receptor
A_3	Adenosine A_3 receptor
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADO	Adenosine 5′-triphosphate disodium salt hydrate
AR	Adenosine receptor
ATP	Adenosine triphosphate
AZ	Active zone
BDNF	Brain-derived neurotrophic factor
BM	Basement membrane
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CaC	Calphostin C
CADO	2-Chloroadenosine
cAMP	Cyclic adenosine monophosphate
cm	Centimeter
CNS	Central nervous system
SC	Schwann cell
DAG	Diacylglycerol
DG	Dystroglycan
DGC	Dystrophin-glycoprotein complex
DMSO	Dimethyl sulfoxide

Dok	Downstream of tyrosine kinase
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
EPP	End-plate potential
GDNF	Glial cell line–derived neurotrophic factor
IP_3	Inositol trisphosphate
K^+	Potassium ion
LAL	*Levator auris longus*
LRP4	Lipoprotein receptor-related protein 4
LTD	Long-term depression
LTP	Long-term potentiation
M_1	Muscarinic 1 receptor
M_2	Muscarinic 2 receptor
M_3	Muscarinic 3 receptor
M_4	Muscarinic 4 receptor
M_5	Muscarinic 5 receptor
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
mEPP	Miniature end-plate potential
MET	Methoctramine
ml	Milliliter (10^{-3}l)
mSC	Myelinating schwann cell
MT3	Muscarinic toxin 3
MuSK	Muscle-specific kinase
Na^+	Sodium ion
nAChR	Nicotinic acetylcholine receptor
NMJ	Neuromuscular junction

NT	Neurotrophin
NTR	Neurotrophin receptor
NT3	Neurotrophin 3
NT4	Neurotrophin 4
O/N	Overnight
OXO	Oxotremorine
P	Postnatal day
PBS	Phosphate buffer saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIR	Pirenzipine
PKA	Protein kinsae A
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral nervous system
SCH-58261	2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo(4,3-e) (1, 2, 4) triazolo(1,5-c)pyrimidin-5-amine
SD	*Sprague-Dawley*
SHC	Src homology 2 domain-containing
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble NSF attachment protein receptor
8-SPT	8-(p-sulfophenyl)theophylline
Syn	Syntaxin
TBE	2,2,2 tribromoethanol
TRICT	Tetramethyl rhodamine iso-thiocyanate
TrkB	Tropomyosin-related kinase B receptor
tSC	Terminal schwann cell
VDCC	Voltage-dependent calcium channel

VDSC	Voltage-dependent sodium channel
Wnt	Wingless-related integration site
YFP	Yellow fluorescent protein
α-BTX	α-bungarotoxin
μg	Microgram (10^{-6}g)
μl	Microliter (10^{-6}l)
μm	Micrometer (10^{-6}m)
μM	Micromolar (10^{-6}M)

ENGLISH

The development of the peripheral nervous system involves an initially exuberant production of neurons and a subsequent activity-dependent reduction in the number of synapses at the neuromuscular junctions (NMJ). This process is called synaptic elimination. At the end of the second postnatal week, each muscle fiber is innervated by a single motoneuron. Muscarinic acetylcholine receptors (mAChR), adenosine receptors (AR) and the tropomyosin-related kinase B (TrkB) receptor may allow the direct competition between nerve endings during synapse elimination through the modulation of acetylcholine release. Here, it has been investigated by confocal microscopy and quantitative morphological analysis the involvement of the individual and synergic or oclusive effect of M_1-, M_2- and M_4-subtypes of mAChRs, A_1 and A_{2A} of ARs and TrkB in the control of the axonal elimination in developing NMJ.

The results show that mAChRs, ARs and TrkB promote axonal disconnection at the beginning of the second postnatal week without affecting the postsynaptic maturation of the nicotinic receptor cluster. In summary, mAChRs, ARs and TrkB delay axonal loss at P7 but accelerate it at P9. In terms of receptor cooperation, M_2 modulates by accelerating the axonal loss the other receptors mainly M_4 and A_1 at P7. The cooperation between M_1, A_1 and A_{2A} receptors promotes axonal loss at P9, whereas the effect of M_2 is independent of the other receptors. M_1 and TrkB receptors work together to increase axonal loss rate at P9 but the effect of M_2 is largely independent of the TrkB receptor.

In conclusion, postnatal synapse elimination is a regulated multireceptor mechanism involving the cooperation of several muscarinic, adenosine subtypes and TrkB receptor that guarantees the monoinnervation of the neuromuscular synapses in the end of the process.

CATALÀ

El desenvolupament del sistema nerviós perifèric implica una inicial exuberant producció de neurones i una posterior reducció dependent de l'activitat del nombre de sinapsis de les unions neuromusculars (NMJ). Aquest procés s'anomena eliminació sinàptica. Al final de la segona setmana postnatal, cada fibra muscular està innervada per una sola motoneurona. Els receptors muscarínics d'acetilcolina (mAChR), els receptors d'adenosina (AR) i el receptor cinasa de tropomiosina B (TrkB) podrien permetre la competició entre terminals nerviosos durant el procés d'eliminació sinàptica mitjançant la modulació de l'alliberament d'acetilcolina. En aquesta tesi s'ha investigat, mitjançant microscòpia confocal i un anàlisi morfològic quantitatiu, el paper dels receptors mAChRs (M_1, M_2 i M_4), dels ARs (A_1 i A_{2A}) i del receptor TrkB en el procés d'eliminació en el desenvolupament de la NMJ.

Els resultats mostren que els receptors mAChRs, ARs i el receptor TrkB promouen una desconnexió axonal al principi de la segona setmana postnatal independentment de la maduració dels receptors d'acetilcolina postsinàptics. En resum, els receptors mAChRs, ARs i el receptor TrkB endarrereixen el procés d'eliminació sinàptica a P7 però l'acceleren a P9. Pel que fa la cooperació entre aquests receptors, M_2 modula, accelerant el procés d'eliminació sinàptica, els altres receptors, sobretot l'acció de M_4 i A_1 a P7. La cooperació entre els receptors M_1, A_1 i A_{2A} promou la pèrdua axonal a P9, mentre que, l'efecte de M_2 és independent dels altres receptors. M_1 i TrkB cooperen per incrementar la pèrdua axonal a P9 però l'efecte de M_2 és independent del receptor TrkB.

En conclusió, l'eliminació sinàptica postnatal és regulada per un mecanisme que depèn de varis receptors, involucrant la cooperació dels diferents subtipus de receptors muscarínics, d'adenosina i el receptor TrkB, els quals garanteixen la monoinnervació de les sinapsis neuromusculars al final del procés.

CASTELLANO

El desarrollo del sistema nervioso periférico implica una inicial exuberante producción de neuronas y, una posterior reducción dependiente de actividad del número de sinapsis en las uniones neuromusculares (NMJ). Este proceso se denomina eliminación sináptica. Al final de la segunda semana postnatal, cada fibra muscular está inervada por una sola motoneurona. Los receptores muscarínicos de acetilcolina (mAChR), los receptores de adenosina (AR) y el receptor quinasa de tropomiosina B (TrkB) podrían permitir la competición entre los terminales nerviosos durante el proceso de eliminación sináptica mediante la modulación en la liberación de acetilcolina. En esta tesis se ha investigado, mediante microscopía confocal y un análisis morfológico cuantitativo, el papel de los receptores mAChRs (M_1, M_2 y M_4), de los ARs (A_1 y A_{2A}) y del receptor TrkB en el del proceso de eliminación en el desarrollo de la NMJ.

Los resultados muestran que los receptores mAChRs, ARs y el receptor TrkB promueven una desconexión axonal al inicio de la segunda semana postnatal independientemente de la maduración de los receptores de acetilcolina postsinápticos. En resumen, los receptores mAChRs, ARs y el receptor TrkB retrasan el proceso de eliminación sináptica en P7 pero lo aceleran en P9. En la cooperación entre estos receptores, M_2 modula, accelerando el proceso de eliminación sináptica, los otros receptores, sobretodo la acción de M_4 y A_1 en P7. La cooperación entre M_1, A_1 y A_{2A} promueve la pérdida axonal en P9, mientras que M_2 es independiente de los otros receptores. M_1 y TrkB cooperan para incrementar la pérdida axonal en P9 mientras que el efecto de M_2 independiente de TrkB.

En conclusión, la eliminación sináptica postnatal está regulada por un mecanismo que depende de varios receptores, involucrando la cooperación de diferentes subtipos de receptores muscarínicos, de adenosina y el receptor TrkB, los cuales garantizan la monoinnervación de las sinapsis neuromusculares al final del proceso.

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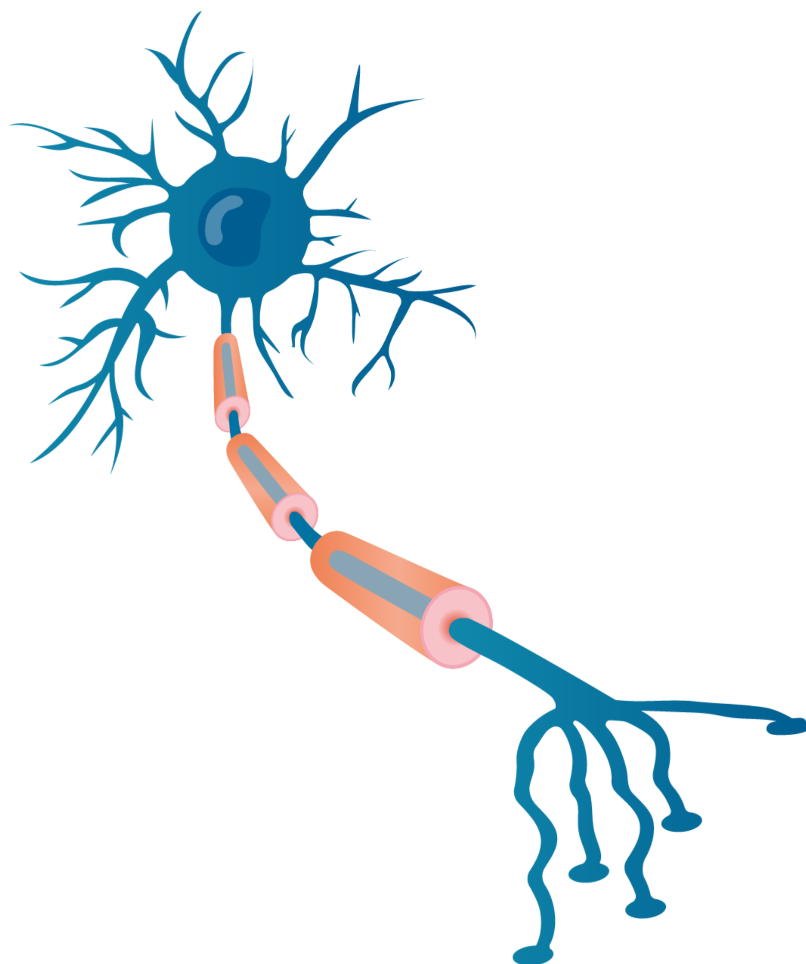


I. INTRODUCTION

1. THE NEUROMUSCULAR JUNCTION: STRUCTURE, FUNCTION AND DEVELOPMENT

The neuromuscular junction (NMJ) is a specialized synapse in the peripheral nervous system (PNS) between a motor nerve terminal (NT) and a skeletal muscle cell (MC). This NMJ is a chemical synapses whose function is to transmit signals from the motoneuron (MN) to the skeletal muscle fiber quickly and reliably, to ensure precise control of skeletal muscle contraction and therefore the movement (Duclert and Changeux, 1995).

The NMJs of all vertebrates display the same basic features: (1) an axon terminal containing the neurotransmitter, acetylcholine (ACh), (2) an overlying Schwann cell (SC) that covers the axon terminal except at the interface of the presynaptic and postsynaptic membranes; (3) an area of synaptic cleft which is lined with the basement membrane (BM) and (4) an invaginated postsynaptic membrane with nicotinic acetylcholine receptors (nAChR) which bind to ACh (Ogata, 1988; Deschenes et al., 1994; Sanes and Lichtman, 1999).

The NMJ is a good synapse model due to its large size and the ease of access experimentally. In concrete, it has contributed greatly to the understanding of the general principles of synaptogenesis and to the development of potential therapeutic strategies for muscular disorders. Aided by the use of suitable animal models, including rodents, zebrafish, *Drosophila melanogaster* and *Caenorhabditis elegans*, studies in the past decade have brought significant progress, not only in identifying components present in pre- and postsynaptic membranes, but also in understanding the mechanisms that underpin NMJ assembly (Keshishian et al., 1996; Schwarz, 2006; Je et al., 2012; Plomp et al., 2015).

Moreover, the NMJ has long been used as a model system for studying the general principles of synapse development (Dennis, 1981; Sanes and Lichtman, 1999, 2001). The development of the NMJ requires an initial guidance of the motor axons toward the specific muscles to be innervated and then the stabilization of the contact. At the end of the NMJ maturation, a muscle becomes innervated by a single axon and persists throughout postnatal life (Sanes and Lichtman, 1999, 2001; Eaton et al., 2002; Pielage et al., 2005).

1.1. STRUCTURE OF NEUROMUSCULAR JUNCTION

The NMJ is composed of three cells or components (Courteaux, 1973; Ogata, 1988; Engel, 1994a; Sanes and Lichtman, 1999) (**figure 1**):

(1) The presynaptic component – the motor nerve terminal of the motoneuron – which is responsible for the synthesis, storage and release of the acetylcholine.

(2) The postsynaptic component – the muscle cell – which contains a high density of nAChRs and other molecules important for the establishment and maintenance of the NMJ.

(3) The glial component – the Schwann cell – which can be teloglia or myelinating Schwann cells according its position along the axon.

In the middle of the presynaptic and postsynaptic component there is the intrasynaptic part – the synaptic basement membrane – which constitutes a structural and functional extracellular matrix and facilitates adhesion between synaptic membranes.

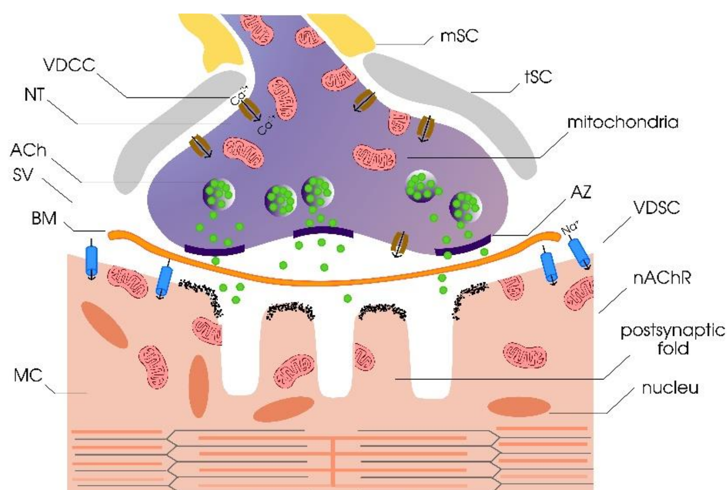


Figure 1. Neuromuscular junction (NMJ) structure: junction between a single-branch axon terminal and the muscle fiber membrane. In purple, the nerve terminal (NT) where active zones (AZ) in purple and synaptic vesicles (SV) with the acetylcholine (ACh) in green are located. Moreover, there are P/Q type voltage-dependent calcium channels (VDCC) to help the fusion of ACh with the NT membrane. In yellow, the myelinating Schwann cell (mSC) and in grey the terminal Schwann cell (tSC) or teloglia. The synaptic cleft contains the basement membrane (BM) in orange. In the muscle cell (MC), there are the nicotinic acetylcholine receptors (nAChR) in black and the voltage-dependent sodium channels (VDSC) in blue.

1.1.1. PRESYNAPTIC COMPONENT

The presynaptic component comprises the motor nerve terminal of a motoneuron. The motoneuron is composed by a cellular body located at the ventral horn of the spinal cord and in cranial nerve nuclei of the brainstem. MNs send their axons to muscles via the ventral roots and cranial nerves. The axon ramifies intramuscularly in collateral axons, each of which ends forming the nerve terminal that contacts a unique muscle fiber. The final branches of NT are thin (about 2 μm) and unmyelinated. Every MN contacting a group of muscle fibers is called motor unit (Hirsch, 2007). To innervate a muscle, several motor units work together. The size of motor units and MNs can differ from one muscle to another. In concrete, the MNs can be small, intermediate or large. Small MNs innervate fewer muscle fibers and generate small contractile forces while larger ones innervate more muscular fibers and generate more powerful forces.

The role of the NT is to release the neurotransmitter that activates the postsynaptic muscle fibers. Neuromuscular synapses in mature vertebrates use the excitatory transmitter ACh, which triggers muscle contraction. ACh is synthesized in motor nerve terminals from the chemical precursor choline and acetyl coenzyme A, facilitated by the acetylcholinesterase (AChE). Most ACh at the NMJ is released in discrete packets of thousands of ACh molecules, called quanta (Fatt and Katz, 1952; Del Castillo and Katz, 1954). Each quantum of transmitter is packaged within a small (50-60 nm diameter), clear, spherical lipid bilayer structure called synaptic vesicle (SV). These vesicles are not present in a random array; instead, there are electron-dense guiding structures which lead vesicles towards membrane sites at which their contents can be released by exocytosis. Synaptic vesicles also contain other compounds, such as adenosine triphosphate (ATP), which are co-released with ACh and are thought to play a particularly important signaling role during development. Slow leakage of unpackaged ACh from motor nerve terminals can also be detected in skeletal muscle cells (called nonvesicular or nonquantal ACh release). However, vesicular release at the NMJ, as at most other synapses, is the dominant mode of transmitter release.

ACh is transferred into synaptic vesicles by the vesicular acetylcholine transporter. This protein exchanges ACh for hydrogen ions, which are concentrated within synaptic vesicles by proton pumps on the vesicular membrane. Both ACh production and loading of ACh into synaptic vesicles depend indirectly on active transport processes that require energy. To provide this energy, motor nerve

terminals contain large numbers of mitochondria (Engel and Franzini-Armstrong, 2004), located near the upper regions of the terminal, away from the muscle-facing surface. Synaptic vesicles within motor nerve terminals can be categorized into three different synaptic vesicle 'pools', based on their release properties. Approximately 1% of vesicles are known as the readily releasable pool (or the release ready pool) because they are primed for immediate release upon firing of a nerve action potential (Richards et al., 2003; Rizzoli and Betz, 2004). A second pool of synaptic vesicles, termed the recycling pool, contains between 5–20% of the total vesicle population. Vesicles from this pool enter the exocytosis-endocytosis cycle under high-frequency physiological conditions (De Lange et al., 2003), so that they can be used repeatedly during sustained activity (Südhof, 2004). The third pool of synaptic vesicles is the reserve/resting pool which represents about 80-90% of all vesicles in the presynaptic terminal (Heuser and Reese, 1973; Delgado et al., 2000). The currently accepted dogma suggests that the vesicles of the reserve pool are only seldomly, if ever, recruited during physiological activity, and are only released by intensive stimulation or when the recycling pool is depleted (Kuromi and Kidokoro, 2000; Ikeda and Bekkers, 2009). Interestingly, a substantial proportion of the vesicles remain unused, even during strong nonphysiological stimulation (Rizzoli and Betz, 2005). A study shows that in central nervous system (CNS) synapses and the NMJ, only 1-5% of all vesicles are recycled (Denker et al., 2011). Hence, the proportion of the reserve pool vesicles that participate in synaptic transmission is still a matter of debate and await further investigations.

Vesicles in the readily releasable pool tend to appear 'docked' at areas on the presynaptic membrane that are highly specialized for the vesicular release of transmitter, called active zones (AZ). Vesicles in the recycling pool are generally located close to, but not docked at, an active zone. In particular, active zones contains high levels of voltage-dependent calcium channels (VDCC) and proteins involved in the fusion of synaptic vesicles with the terminal plasma membrane (Zhai and Bellen, 2004). When an action potential reaches the nerve terminal, VDCC of the P/Q type (N-type channels may also localize to the presynaptic membrane) are activated, calcium enters the presynaptic terminal, and the local calcium concentration rises significantly, triggering the fusion of the synaptic vesicle membrane with the plasma membrane of the nerve terminal (Robitaille et al., 1993; Sugiura et al., 1995; Day et al., 1997; Rizo and Rosenmund, 2008). Moreover, the presynaptic terminal contains muscarinic acetylcholine receptors (mAChR) which have functions in the maintenance and synaptic efficacy (Ganguly and Das, 1979;

Abbs and Joseph, 1981; Wessler et al., 1987; Arenson, 1989; Caulfield and Birdsall, 1998; Nathanson, 2000; Parnas et al., 2000; Garcia et al., 2005), adenosine receptors (AR) (Garcia et al., 2013; Oliveira et al., 2015) and neurotrophin receptors (NTR) such as tropomyosin-related kinase B (TrkB) receptor (Gonzalez et al., 1999; Garcia et al., 2010e).

1.1.2. SYNAPTIC CLEFT AND BASEMENT MEMBRANE

The synaptic cleft is the space between the nerve terminal and the postsynaptic membrane, of approximately 50 nm wide in the NMJ.

The basement membrane, also called basal lamina, is a complex structure which can be further subdivided into an internal basal lamina layer connecting to the sarcolemma and an external reticular lamina layer (Patton, 2003; Sanes, 2003). The BM envelops the entire muscle fiber including the synaptic cleft between the nerve terminal and endplate, but the reticular lamina is excluded from the synaptic cleft of NMJs (Sanes, 2003). The BM is made up largely of collagen IV molecules, laminins, and other non-collagenous proteins including entactin/nidogen, perlecan, and fibronectin (Timpl et al., 1979; Timpl and Brown, 1996; Patton, 2003; Sanes, 2003). Also, agrin, neuregulin and ACh-receptor inducing activity which are involved in the formation of the NMJ, and neurotrophin substances like fibroblast growth factor (FGF) (Wood and Slater, 2001; Mis et al., 2013). BM have three main roles in the NMJ (Patton, 2003). First, BM contribute to keep the structural integrity. Second, BM promote cell migration during development, and cellular polarity, stability, and intercellular interactions in adults. Third, BM possess potent signaling components, which variously promote proliferation, survival, and differentiation.

The basement membrane contains an important enzyme, the AChE (Salpeter, 1987; Rotundo, 2003), which is a type-B carboxylesterase needed for the rapid breakdown and inactivation of released ACh. The enzyme is secreted from the muscle but remains attached to it by thin stalks of collagen fastened to the BM. When ACh is released from the active zone by an action potential, approximately 10% of released ACh will bind to AChE, whereas the remaining 90% will diffuse through the basal lamina to the muscle membrane to bind to nAChRs. When ACh molecules dissociate from nAChR, they diffuse into the synaptic cleft and bind to AChE, which will once more be available to hydrolyze free ACh, affer having hydrolyzed the ACh that was bound to it earlier. The rapid action of AChE prevents

ACh from binding more than once to nAChRs. The breakdown of ACh by AChE at the NMJ is a more efficient means of stopping transmission than what occurs at most central nervous system synapses, where the termination of transmission is most often achieved by the reuptake of transmitter into the presynaptic terminal. The efficiency of AChE ensures that transmission is restricted to a short time window within an area near the site of transmitter release, thus contributing to the temporal and spatial precision of skeletal muscle control.

1.1.3. POSTSYNAPTIC COMPONENT

A muscle fiber or myocyte is a long and cylindrical multinucleated cell where the nerve terminal forms the synaptic contact in the middle of it. Muscle cells are red due to the large number of mitochondria and myoglobin that they have, which provide the energy and oxygen requirements of contraction.

The muscle fiber is enveloped by a basal lamina and a special muscle cell membrane called the sarcolemma. The sarcolemma acts as a physical barrier against the external environment and facilitates signaling to the fiber. The cytoplasm of a muscle fiber is called sarcoplasm. Most of the sarcoplasm is filled with myofibrils. Myofibrils are composed of actin, myosin and other proteins that keep them together. These proteins are arranged in thin and thick filaments, the myofilaments, which are repeated throughout the myofibrils into sections called sarcomeres – the smallest functional unit of muscle fiber. Thick filaments are composed of myosin and thin filaments are composed of actin. The striated appearance of the myofibril is achieved by the combination of actin filaments that form the light band (I band) and myosin filaments that form the dark band (A band). The sarcomeres are separated by Z-line (also known as the Z-disc or Z-band), which are aligned between myofibrils of the same cell. At the junction overlap between the A and I bands of the sarcomere are located the T-tubules, which together with a pair of terminal cisternae (bulbous enlarged areas of the sarcoplasmic reticulum) form an arrangement called a triad (Padykula and Gauthier, 1970).

Another important part in the postsynaptic component are nAChRs which are located on a specialized region of skeletal muscle fibers called the motor endplate. This structure is recognizable by invaginations in the postsynaptic plasma membrane, known as junctional folds. These are positioned under the active zones

of presynaptic nerve terminals. In the area where the contact takes place, the membrane forms a concavity named primary fold. To increase further the contact surface, there are invaginations of the membrane in the primary fold of the fiber which receive the name of synaptic secondary folds (Salpeter, 1987). At the bottom of the secondary folds, there are voltage-dependent sodium channels (VDSC), together with the cytoskeleton fibers, microtubules and microfilaments which maintain the structure of the primary and secondary folds and also keep the nAChRs anchored to the membrane on the top of the secondary folds (Flucher and Daniels, 1989). The crests of the folds are very defensibly packed with nAChRs at a density of about 10.000 per μm^2 (Salpeter and Loring, 1985).

The nicotinic receptors are considered cholinergic receptors, since they respond to acetylcholine when is released from the presynaptic terminal. Each nAChR has two binding sites for ACh. The function of the endplate nAChR depends on five subunit proteins (development: $\alpha_2\beta\delta\gamma$; adult: $\alpha_2\beta\delta\varepsilon$) that combine to form the pentameric unit. Each subunit of this receptor has a characteristic cys-loop, which is composed of a cysteine residue followed by 13 amino acid residues and another cysteine residue. The two cysteine residues form a disulfide linkage which results in the cys-loop receptor that is capable of binding ACh (Engel, 1994b, 1994c). When ACh binds, this receptor undergoes a conformational change that allows ions to pass (influx of sodium ions (Na^+) into the muscle and output of postassium ions (K^+)) during roughly 1-2 ms. The ion pass triggers a depolarization of the postsynaptic membrane which, if it exceeds the threshold, ends up evoking contractile activity.

1.1.4. GLIAL COMPONENT

Glial cells are widely distributed throughout the nervous system and all peripheral axons which, whether myelinated or not, are covered with Schwann cells (SC). There are two types of SCs: myelinating and nonmyelinating. SCs play an important role in synaptic function and formation (Corfas et al., 2004; Kettenmann and Ransom, 2005). Recent findings are forcing that glial cells play multiple active roles at synapses. It is important the concept of the ‘tripartite’ synapse, which includes synapse-associated glial cells as active participants in the formation, function, maintenance and plasticity of pre- and postsynaptic components (Araque et al., 1999).

Preterminal or myelinating Schwann cells (mSC) wrap around axons of motor and sensory neurons to form the myelin sheath. The myelin sheath is composed of compacted layers of the SC membrane, which is predominantly lipid, but contains several proteins that take on key roles in maintaining the structure and compaction of the myelin and adhesion of the sheath to the axon. One SC myelinates one axon. The axons with a diameter exceeding 1-2 µm release myelination signals, which stimulates myelin production. The myelin enwraps the axon in segments that are separated by nodes of Ranvier. Nodes of Ranvier are unmyelinated, critical to the functioning of myelin and highly enriched in ion channels, allowing them to participate in the exchange of ions required to regenerate the AP (Black et al., 1990; Rasband and Trimmer, 2001). SCs act as an electrically insulating sheath, thus, signals are propogated along the axon in a series of jumps from node to node, in a process called saltatory conduction. In this manner, the need for action potential regeneration at every point of axonal membrane is eliminated, thereby reducing metabolic requirements for neural activity.

The second group, called terminal Schwann cells (tSC) or teloglia, send out processes that cover unmyelinated nerve terminals on the nonsynaptic facing side. tSCs play a significant role in the development, function, maintenance, and regeneration of NMJs (Auld and Robitaille, 2003; Auld et al., 2003; Kang et al., 2003; Corfas et al., 2004; Rousse et al., 2010; Ko and Robitaille, 2015). For example, during development, tSCs have been shown to promote synapse formation and the clustering of nAChRs. Furthermore, tSCs are very closely opposed to motor nerve terminals and there is mounting evidence for bidirectional signaling between nerve terminal and tSC. ACh released from motor nerve terminals can induce calcium waves in tSCs (Robitaille et al., 1996; Day et al., 1997), and conversely, tSCs can affect transmitter release from MNs, suggesting that tSCs play an important role in modulating synaptic function (Auld and Robitaille, 2003). Moreover, tSCs contain mAChRs that binds the ACh released from the nerve terminal (Robitaille et al., 1997; Georgiou et al., 1999; Rochon et al., 2001; Todd et al., 2007), purinergic receptors (Robitaille, 1995) and L-type VDCCs (Robitaille et al., 1996). Recent research has identified a fourth specialized cell type at the NMJ, named the kranocyte. Kranocytes form a cap over motor nerve terminals and proliferate extremely rapidly after nerve injury, suggesting that they may perform as yet undescribed functions at damaged NMJs (Court et al., 2008).

1.2. FUNCTION OF NEUROMUSCULAR JUNCTION

1.2.1. NEUROMUSCULAR TRANSMISSION

The NMJ is a chemical synapse, meaning that the motoneuron and skeletal muscle cell are not physically connected to one another. Instead, action potential firing in MN causes the release of chemical messengers that diffuse across the basement membrane to initiate contraction (Ivan HK and Etherington, 2011).

From a broad perspective, neuromuscular transmission occurs by a fairly simple and straightforward mechanism (**figure 2**). An action potential is conducted down the somatic MN and arrived at NT (**figure 2A**). The reversal in electrical polarity at the synaptic cleft causes an opening of VDCC with a subsequent abrupt increase in intracellular calcium concentration (**figure 2B**) (Cohen-Cory, 2002). This increased calcium concentration triggers a cascade of intracellular signaling events leading neurotransmitter-containing vesicles to migrate to the surface of the nerve, rupture and discharge ACh into the cleft separating nerve from muscle (**figure 2C**). To enable the neurotransmission, apart from calcium ions (Ca^{2+}) influx induced by depolarization, is also necessary the SNARE (Soluble NSF Attachment Protein Receptor) complex assembly which implies a number of proteins found in the membranes and the cytoplasm of the MN (Rizo and Xu, 2015). The proteins that integrate the SNARE complex are divided in two groups: the first formed by Synaptobrevin-2, a protein that is found in the membrane of synaptic vesicles and the second group composed of proteins that are associated with the membrane of the nerve terminal, Synaptosomal-Associated Protein 25 (SNAP-25) and Syntaxin-1 (Bajjalieh and Scheller, 1995; Südhof, 1995, 2004). Nevertheless, after many studies, it has been confirmed that the SNARE itself is not enough for the fusion to take place; instead, some more proteins are needed to allow the synaptic membrane fusion. The best known of this 'complementary proteins' are Synaptotagmin, Complexin, Munc13 and Munc18-1 (Jahn and Fasshauer, 2012).

Once the exocytose of the neurotransmitter ACh has taken place, it spreads through the synaptic cleft and binds the nAChRs in the postsynaptic membrane (**figure 2D**). nAChRs respond by opening their channels for influx of Na^{+} into the muscle to depolarize the muscle. Then, the potassium channels impulse the K^{+} out of the cell and generate, this way, the repolarization (**figure 2E**). This procedure generates a change in the polarity among inside and outside the neuron that starts at the soma and travels by the axon until it reaches the nervous terminal. The result is a modified end-plate potential (EPP) which corresponds to a

local membrane depolarization of the muscle fiber. This local depolaritzation, activate the postsynaptic VDSCs which are located in the deeper part of the secondary folds (Flucher and Daniels, 1989). If enough of these sodium ions enter the muscle fiber to raise it from its resting potential of -95 mV to about -50 mV, they trigger an action potential that spreads throughout the fiber (**figure 2F**). This potential travels first along the surface of the sarcolemma and then arrived to myofibrils. To reach the myofibrils, some of which are located deep in the muscle fiber, the muscular action potential travels through the T-tubule system. The action potential activates VDCCs in the T tubule membrane. Calcium channels in the T tubules lie in close apposition to calcium release proteins in the sarcoplasmic reticulum. Conformational changes in T tubule calcium channels induced by depolarization are transmitted by a direct protein-protein interaction to the sarcoplasmic reticulum calcium release protein, triggering the release of calcium from the sarcoplasmic reticulum into the muscle cytoplasm. The rise in intracellular calcium activates contractive protein. The muscle contraction is a complex procedure that implicates actin filaments, also known as thin filaments and myosin. When the impulse reaches the muscle fibers, it stimulates a reaction between actin and myosin in the sarcomere. The electric pulse stimulates the heads of myosin fibers to attach the actin filaments, which then pulls towards the center of the sarcomere. As this process takes place in all the sarcomeres, all get shortened at the same time. The relaxation of the muscle fiber is associated with the return of the calcium ions to the sarcoplasmic reticulum.

After the depolarization of the muscle membrane, ACh immediately detaches from the receptor and is destroyed by the nearby AChE located in the synaptic cleft (Slater, 2008). This enzyme serves two purposes, namely it (1) permits only a small proportion of ACh released to stimulate receptors and their channels on the sarcolemma of the endplate and (2) creates breakdown products, acetyl and choline, which are taken up by the nerve terminals and utilized in the resynthesis of ACh.

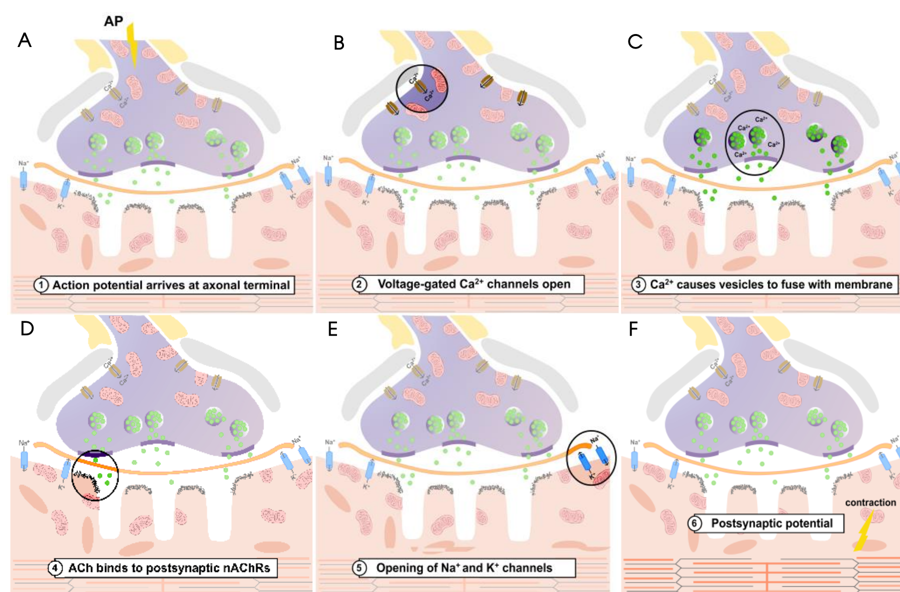


Figure 2. Synaptic transmission at the neuromuscular junction. A. An Action Potential (AP) is conducted down the somatic motoneuron (MN) down to the presynaptic terminal. **B**. The electrical polarity at the presynaptic terminal causes an opening of voltage-dependent calcium channels (VDCC). **C**. The entry of Ca^{2+} into the presynaptic terminal causes the exocytosis of the neurotransmitter acetylcholine (ACh). **D**. The ACh diffuses across the synaptic cleft and binds to nicotinic acetylcholine receptors (nAChR) on the membrane of the skeletal muscle. **E**. Activation of the nAChRs causes an opening of 'ligand-gated' Na^{2+} ion channels and K^{+} ion channels. **F**. As sodium ions flow into the skeletal muscle, it depolarizes to the threshold potential, triggering an AP. As the AP spreads along the cell, it causes the muscle cell to contract.

1.3. DEVELOPMENT IN NEUROMUSCULAR JUNCTION

The mechanisms that govern synapse formation and elimination are fundamental to understand the neural development and plasticity. The NMJ development comprises the formation and organization of the three NMJ cells – motoneuron, muscle fiber, and Schwann cell. Neuromuscular transmission requires that pre- and postsynaptic components develop in tight register with one another, implying reciprocal interactions between them. Schwann cells are also an indispensable structural and functional component of the synapse. They modulate synaptic transmission and also play important roles in synapse formation and maintenance.

There are two main stages in the development of the NMJ: embryonic stage and postnatal stage, the latter involving the synapse elimination process.

1.3.1. EMBRYONIC STAGE

All three cells of the NMJ travel long distances to meet at the synapse. In the presynaptic site, axons of the MNs proliferate in the ventricular zone of the neural tube and become postmitotic. Motor axons exit the central nervous system through ventral roots or cranial nerves, then run long distances through peripheral nerves to muscles (Leber et al., 1990). As motor axons extend toward developing muscle, muscle cells are themselves undergoing differentiation. Muscle fibers develop from progenitor cells in the paraxial mesoderm (reviewed in Brand-Saberi et al., 1996). In the trunk, paraxial mesoderm is segmented into somites that lie on either side of the neural tube. Myogenesis involves two waves of precursor proliferation: the first wave (embryonic day 9.5 to E14.5 in mice) involves muscle progenitors that proliferate in the somites and migrate to their final location where most of them differentiate and fuse to form immature embryonic muscle fibers, called primary myotubes. In this stage, the presence of calcium ions is critical. A fraction of these progenitors, however, do not fuse and give rise to fetal myoblasts that continue to proliferate and either fuse with primary myotubes or, using them as a scaffold, begin to fuse among each other to form a new population of secondary myotubes (second wave) between E15 and E17 (Cossu and Biressi, 2005). Once the myotubes are developed, they start to synthetize the contractile apparatus. Initially, the actin and myosin proteins arrange their skeleton randomly but quickly they polarize and aggregate longitudinally along the myotube. This process is called myofibrillogenesis. Although myotubes may have a functional contractile apparatus, they are not considered myocytes until they are innervated by a MN. This is because although the postsynaptic component is almost entirely developed, it does not work without innervation. After the muscle is innervated, angiogenesis oxygenates the muscle to stand contraction.

Schwann cells, the third component, are derivatives of the neural crest, which arises from the dorsal margin of the neural tube. Both SCs and motor axons traverse the rostral halves of the somites, from which they derive their segmental arrangement. It is probably within or near the somites that SCs become associated with motor axons. Thereafter, SCs follow motor axons through the periphery and into muscles. During this journey, the axons provide both migratory guidance and

mitogenic stimulation for the SCs (reviewed in (Mirsky and Jessen, 1996) at the same time as SCs might guide motoneuron growth cones (Reddy et al., 2003).

Once the motor axon's growth cone contacts a newly formed myotube, synaptic transmission commences quickly. Initially, however, the efficacy of transmission is extremely low, reflecting the absence of both pre- and postsynaptic specializations. Over a period of about a week, a fully functional synapse forms in which both nerve and muscle are differentiated (**figure 3A**).

On the presynaptic differentiation, growth cones can release neurotransmitter in response to electrical stimulation even before they make contact with muscle fibers (Hume et al., 1983; Young and Poo, 1983). Moreover, spontaneous and evoked neuromuscular transmission begin within minutes after nerve contacts muscle (Kidokoro and Yeh, 1982; Chow and Poo, 1985; Xie and Poo, 1986; Evers et al., 1989). However, synapses are initially very weak, not only because nAChRs density is low, but also because very little transmitter is released (Kullberg et al., 1977; Nakajima et al., 1980). Over the subsequent days, as the postsynaptic membrane matures, the presynaptic terminal also differentiates dramatically.

Nerve terminals at newly formed synapses are simple bulbous enlargements (Linden et al., 1988). They bear few synaptic vesicles and no ultrastructurally recognizable active zones. Subsequently, synaptic vesicles increase in number, and cytoskeletal elements characteristic of the axon are lost. Active zones appear, vesicles become clustered at the active zones, and the nerve terminal becomes polarized. These changes, accompanied by a parallel increase in synaptic volume and area, underlie large increases in the frequency of spontaneous exocytosis and the number of vesicles that release neurotransmitter in response to stimulation (Dennis, 1981).

At the same time as presynaptic component is differentiating, nAChRs form few clusters in the primary myotubes (Feng et al., 1999; Lin et al., 2008). Days after, neuromuscular contacts form in the center of the muscle, as indicated by the close apposition of nerve terminals with clusters of nAChRs and AChE (Lin et al., 2001, 2008; Yang et al., 2001). However, this central band (~200 μm wide) contains many nAChR clusters that are not contacted by any nerve. Together with the fact that nAChR clusters also form in mouse mutants lacking phrenic nerves (Lin et al., 2001; Yang et al., 2001), this suggest that receptor clusters are formed by a nerve-independent patterning mechanism. Hence, these aneural nAChR clusters have been termed `prepatterned'. Between E13.5 and E15.5, the fraction of nAChR

clusters contacted by the nerve increases from <10% to close to 50% (Lin et al., 2008). By E18.5, only innervated nAChR clusters remain and have grown in size, while aneural clusters have disappeared (Lin et al., 2001; Yang et al., 2001). In this early phase, a single postsynaptic nAChR cluster may be contacted by up to 10 motor axons at birth (Tapia et al., 2012). Importantly, the majority of nAChRs at these stages have a distinguished arrangement known as the 'fetal' subtype, in which this ion channels are composed of four different subunits, termed α, β, γ, and δ in the stoichiometry $\alpha_2\beta\delta\gamma$. Also they are characterized by an open burst duration of ~4–5 ms (Mishina et al., 1986).

In summary, motoneurons, muscle cells, and Schwann cells acquire their identities before synapses form and then they meet to form an efficient synapse.

1.3.2. POSTNATAL STAGE

The NMJ undergoes dramatic changes in structure and function during the first few postnatal weeks. At birth, each muscle fiber is innervated by multiple motoneurons (**figure 3B**) and all but one motoneuron input are gradually eliminated over the course of several weeks (**figure 3C**). This process is called synapse elimination (see in more detail in section 2) (Redfern, 1970; Brown et al., 1976; Slater, 2008; Tapia et al., 2012). Initially, all of the axons that innervate each muscle fiber are intertwined atop an elliptical, uniform plaque of nAChRs. Then, as inputs are eliminated, spots of low nAChR density appear as perforations within the plaque. Sculpting continues until the postsynaptic apparatus acquires a pretzel-like form (Nyström, 1968; Steinbach, 1981; Slater, 1982a; Balice-Gordon and Lichtman, 1993). First, the membrane becomes depressed into shallow gutters beneath the nerve terminals. Subsequently, gutters invaginate to form junctional folds, which invariably lie directly across from the active zones in the nerve terminal.

Schwann cells also mature postnatally. At birth, tSCs form a loose cap over groups of axonal boutons at each synaptic site, whereas each terminal bouton is separately capped in adults (Hirata et al., 1997). Perhaps more importantly, mSCs are present in embryos, but do not form myelin until postnatally. In addition, both mSCs and tSCs are acutely dependent on axonal contact for their survival in neonates, but become relatively nerve-independent postnatally (Trachtenberg and Thompson, 1996).

To sum up the development of the NMJ, synaptic transmission begins soon after contact; indeed, growth cones release small amounts of transmitter even before the contact has been made. The efficacy of this transmission is low due to the absence of pre- and postsynaptic specializations. Growth cones of other motor axons soon converge at the same site formed by the initial contact and begin forming new synapses, resulting in the polyneuronal innervation of muscle fibers. The maturation of the NMJ involves different changes in pre- and postsynaptic component. At the presynaptic component motor axons become myelinated and some of them are eliminated through synapse elimination process. At the postsynaptic component, the most important change is in nAChRs (see in section 2). Coordinate expansion of the nerve terminal and muscle fiber lead to growth of the NMJ without major change in synaptic geometry. The structural maturation of the NMJ is largely completed 3-4 weeks after birth (Slater, 1982a), being considered mature around P30 (**figure 3D**). Mature NMJs persist for the life of the animal. This maintenance include different molecular mechanisms and imply the three cells of the NMJ.

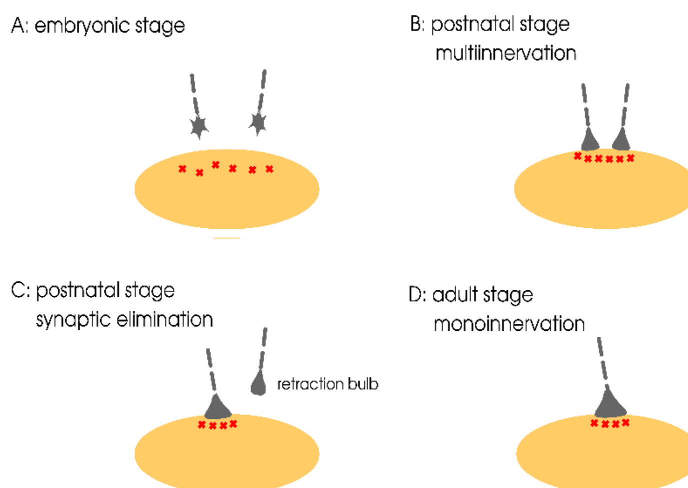


Figure 3. Development of neuromuscular junction: overview of the different stages of development. **A**: at embryonic stage the three neuromuscular junction (NMJ) cells (motoneurons, muscle cells, and Schwann cells) acquire their identities before synapses form and then they meet to constitute an efficient synapse. **B**: after birth, the NMJ undergoes dramatic changes in structure and function during the first few postnatal weeks. Terminals from competing inputs become segregated and the plaque acquires perforations. **C**: during the postnatal stage, the synapse elimination process occurs where only one motoneuron remains in the synaptic site whereas the other contacts are eliminated forming a retraction bulb. **D**: in the mature muscle, each muscle fiber is innervated by one motoneuron.

2. SYNAPSE ELIMINATION PROCESS

In the development of both central and peripheral nervous systems, synapse formation generates some connections that exist only transiently. It is difficult to do such detailed analyses in the CNS, because the small size of synapses, heterogeneity and abundance of synaptic inputs to each neuron, and the complexity of synaptic organization (Lohof et al., 1996; Kano and Hashimoto, 2009). In this respect, in the visual system, thalamocortical axons disconnect from cortical layer IV cells (Hubel et al., 1977); in the cerebellum, climbing fibers disconnect from Purkinje cells (Daniel et al., 1992; Hashimoto and Kano, 2005); in autonomic ganglia, preganglionic inputs disconnect from ganglion cells (Lichtman, 1977); and at the neuromuscular junction, motor axons disconnect from muscle fibers (Benoit and Changeux, 1975; O'Brien et al., 1978). In each of these areas, elaboration of synapses by the remaining axon or axons also occurs. Thus, while some inputs are being eliminated, others are becoming stronger.

Synapse elimination at the skeletal neuromuscular junction is currently the best studied model. In the NMJ, synapses are large and can be visualized by labeling axons and/or postsynaptic structures, which allow us to monitor the changes over days and weeks.

2.1. PRESYNAPTIC COMPONENT: FROM POLY- TO MONONEURONAL MUSCLE FIBER INNERVATION

Most vertebrate muscle fibers are innervated by more than one motor axon during developmental period. All inputs but one are withdrawn during early postnatal life in a process called synapse elimination (**figure 4)**. Nevertheless, the size of the endplate and the numbers of nAChRs, junctional folds, and active zones all increase during this period.

Although neuromuscular synapses are established before birth (in rodents about a week before), the transition from multiple to single axonal innervation typically does not occur until the first several postnatal weeks, perhaps indicating a role for normal function in the elimination process. At birth each muscle fiber possesses a single well-circumscribed oval- or plaque-shaped site where multiple axons converge (terminal segregation) (**figure 4**, step 1). At this time, the strengths of the competing axons are often quite similar and do not suggest which input will

eventually be removed. Possibly the winners and losers are not yet determined at birth, requiring competition in early postnatal life for the outcome to emerge. Over the first several postnatal weeks, the number of junctions that are occupied by more than one axon gradually declines. In some muscles, the rate of loss is sigmoidal, starting slowly and reaching a peak of ~10% per day before trailing off once nearly all of the junctions are innervated by one axon (reviewed by Jansen and Fladby, 1990). Thus, there is gradual loss of multiple innervation, so that some neuromuscular junctions remain multiply innervated for nearly two postnatal weeks, whereas other junctions lose their multiple innervation in several days. This variation in the time to completion is probably closely related to the fact that the loss of multiple innervation is also gradual in another sense: each individual junction gradually loses multiple innervation (**figure 4**, step 2).

Koirala and Ko, 2004 using time-lapse imaging of fluorescently labeled axons and serial electron microscopy showed that axons at neuromuscular junctions are removed by an unusual cellular mechanism. As axons disappear, they shed numerous membrane bound remnants. These 'axosomes' contain a high density of synaptic organelles and are formed by engulfment of axon tips by Schwann cells. Moreover, Turney and Lichtman, 2012 showed that laser removal of one axonal input to a multiply innervated neuromuscular junction invariably leads to takeover of the synaptic site by the remaining input. So, the axons grew to occupy vacant sites even when they appeared to be in the process of withdrawing at the time the sites were vacated. The axons were stimulated to grow even in situations when the muscle fiber was still active. This combination of synaptic vacancy and the axonal takeover it induces allows to explain a range of complex phenomena associated with synapse elimination.

During this period, a progressive strengthening of some inputs and weakening of others is evident functionally as well as structurally within individual junctions (Walsh and Lichtman, 2003; Bloch-Gallego, 2015). Quantal content becomes increasingly disparate among competing inputs (Colman et al., 1997), and the density of nAChRs beneath some inputs is sharply reduced (Balice-Gordon and Lichtman, 1993), leading to a reduction in their quantal efficacy. The progressive loss of presynaptic terminals (Balice-Gordon and Lichtman, 1993; Gan and Lichtman, 1998) decreases the effective area for neurotransmitter release by weakened inputs. Cycles of functional weakening and structural loss continue until all of the sites innervated by weakened inputs are eliminated, and the losing axons permanently withdraw from the junction presenting a retraction bulb and atrophy

of the nerve terminal (Riley, 1977, 1981; Balice-Gordon and Lichtman, 1993; Gan and Lichtman, 1998). The loss of territory by an axon is contemporaneous with and may be related to the transformation from an oval, nAChR-rich plaque to a pretzel-shaped structure (Balice-Gordon and Lichtman, 1993). It is not known, however, what properties of an axonal branch or its environment determine its destiny in these competitions.

An important consequence of the process of synapse elimination is that each MN ends up innervating muscle fibers with similar properties. Clear signs of functional homogeneity of the muscle fibers within motor units are seen in mice two weeks after birth, as synapse elimination nears completion but before the distinctive patterns of activity of different motor units are clearly developed (Fladby and Jansen, 1988). It therefore seems unlikely that differences in activity patterns between MNs play a decisive role in either survival selection or in matching the properties of MNs to the muscle fibers they innervate.

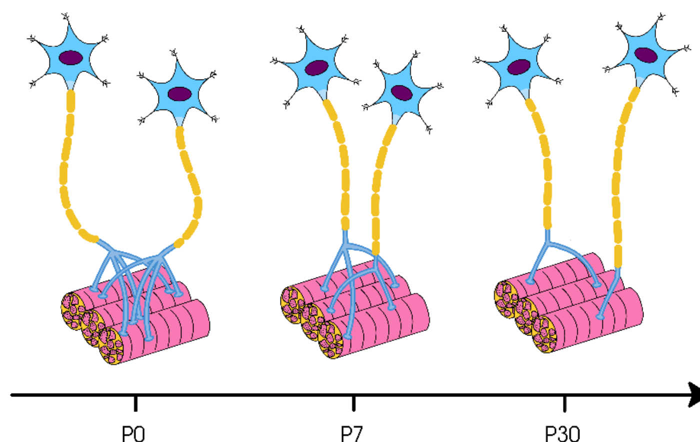


Figure 4. Critical periods of neuromuscular junction development focus on the synapse elimination. At birth (P0), each muscle fiber is innervated by several axons of motoneurons (MN). Postnatally, a critical step with the elimination of about half multi-innervation occurs between P0 to P7. The process of synapse elimination is achieved when all muscle fibers are singly innervated (P30).

It is not known, what are the factors which determine the synaptic elimination process. Indeed, several different local mechanisms have been proposed to explain what drives this process forward. One idea is an activity-based model requiring a cascade of anterograde, intersynaptic and retrograde signals (Jennings, 1994). It has been demonstrated that asynchronous activity leads to synapse elimination whereas synchronous activation of the postsynaptic cell is

unable to cause synapse loss. This idea was tested by replacing the normal activity patterns with synchronous volleys of stimulation to the nerve regenerating to a muscle (Busetto et al., 2000). The result was clear: pure synchronous axonal activation prevented synapse elimination during the reinnervation process. Thus, the mechanism of synapse elimination appears to depend less on the presence of activity per se and more on the relative activity patterns of the competing inputs. Moreover, it is known that the initial matching of nerve and muscle cells is achieved by a molecular recognition system that involves activity-dependent expression of surface and/or diffusible molecules that interact to promote survival of the most compatible pairs at each developing NMJ (Nguyen et al., 1998; Gonzalez et al., 1999). Such a mechanism could depend on activity and other molecules that could promote survival and the outcome of the competition.

In laboratory in which this thesis has been performed, the process of synaptic elimination has been extensively studied by immunohistochemistry (e.g. Lanuza et al., 2002, 2003 as well as this thesis) and by intracellular electrophysiology (e.g. Santafé et al., 2003, 2004, 2009). The second technique, allows the recording of depolarizations across the myocyte plasmalemma in the shape of EPP or miniature end-plate potentials (mEPP). EPPs are caused by the increase in acetylcholine in the synaptic cleft due to the coordinated exocytosis of multiple vesicles, hence their denomination 'evoked'. In contrast, mEPPs correspond to the spontaneous release of ACh vesicles in the absence of an action potential, therefore being the smallest depolarization which can be induced in a muscle. Multiply innervated synapses present multiple EPPs that can be distinguished. Consequently, electrophysiology is useful when it comes to determine the functional changes that undergo the different terminals during synapse elimination and how different molecules can modulate the neurotransmitter release of two motor nerve terminals competing on a dually innervated endplate.

During neonatal synaptic competition, dual junctions can provide a simple model for studying the structural, molecular and functional differences between the two axons. Whereas one axon wins the competition and consolidates, the other one is eliminated (this is generally the small ending because of a progressive reduction in synaptic area occupation; (Kopp et al., 2000; Santafé et al., 2001, 2002). In dually innervated fibers, a second EPP can appear after the first one when the intensity of the electrical stimulus is increased. This compound EPP is built by recruiting two axons. The EPP amplitude of the second axon response is calculated by subtracting the first EPP amplitude from the compound EPP. The

lowest and highest amplitudes are known as 'the small EPP' and 'the large EPP', respectively. In previous studies, the nerve terminals that produce these synaptic potentials have been refered as the 'weak' and 'strong' endings (Santafé et al., 2003, 2004, 2009a).

2.2. POSTSYNAPTIC COMPONENT

At the same time axons are gradually losing their synaptic hold on postsynaptic cells, there is a change in the postsynaptic component. It has been described that the ability of the postsynaptic cell to change at locations where nerve terminals will later be removed raises the possibility that alterations in the postsynaptic cell may be the cause of nerve terminal removal (Lichtman and Colman, 2000).

The well-known important change in the structure of the postsynaptic apparatus occurs in the nAChRs. An important conversion of nAChRs from an embryonic to an adult form happens: fetal nAChRs ($\alpha_2\beta\delta\gamma$) at the NMJ are gradually replaced by the adult subtype of nAChRs ($\alpha_2\beta\delta\varepsilon$) over a period of ~10 days and are further concentrated to high density (Brenner and Sakmann, 1978; Matthews-Bellinger and Salpeter, 1983; Jaramillo et al., 1988). The adult nAChRs contain the nAChR ε-subunit instead of the γ-subunit, which results in shorter open burst durations (~1 ms) but increased conductance for Na^+, K^+, and Ca^{2+} (Mishina et al., 1986).

Other postnatal changes include the metabolic stabilization of synaptic nAChRs: nAChRs and VDCCs segregate into discrete domains, the formation of subsynaptic infoldings of the membrane (Matthews-Bellinger and Salpeter, 1983; Marques et al., 2000); the recruitment of muscle nuclei, called fundamental myonuclei (up to 6 in adult mouse muscle; Kues et al., 1995), to the cytoplasmic region below the synaptic membrane; and an increase in size and density of synaptic nAChR clusters (10- fold from approximately 1000/μm^2 to more than 10000/ μm^2) accompanied by changes of their shape from plaques to pretzels (Steinbach, 1981; Slater, 1982a; Sanes and Lichtman, 1999; Jessen and Mirsky, 2005). The breaking up of a plaque-shaped synaptic nAChR cluster into a pretzel of the same area may be important for lowering the distance for which the synaptic current needs to flow across the high electrical resistance along the synaptic cleft, thus maintaining high driving force for the synaptic inward current. Finally, as the

synaptic folds mature, nAChRs become concentrated at their crests (Fertuck and Salpeter, 1974) and $Na_V1.4$ in their troughs (Flucher and Daniels, 1989; Stocksley et al., 2005).

The gradual transition from multiple to single innervation seems to be related to local differences in the postsynaptic area. The postsynaptic receptor density shows signs of disassembly as receptors are removed from the synaptic sites. Postsynaptic sites continue to lose nAChR density until they disappear entirely. By two postnatal weeks, an adult pretzel-like shape is obtained at each neuromuscular junction. In some muscles at least, the junction continues to grow by intercalary enlargement pre- and postsynaptically for many months (Balice-Gordon and Lichtman, 1990; Lichtman and Colman, 2000). In this thesis, the postsynaptic changes of nAChRs at NMJs have been divided into six stages based on criteria from previous studies of developing mammalian NMJs (Steinbach, 1981; Slater, 1982a; Bewick et al., 1996; Lanuza et al., 2002). The following maturation stages (MS1 to MS6) were defined (**figure 5**):

- MS1. Uniform nAChR organized in an oval or round plaque with blurry boundaries seen at the majority of NMJs at birth. The reconstructions indicate that these plaques are slightly concave relative to the nearby muscle fiber membrane, typically in the shape of a spoon. Within the plaque the receptors were arranged in patches that resembled cobblestones (2–3 µm in diameter) that were separated by well-defined dark bands, presumably because of nascent folding of the postsynaptic membrane.
- MS2. nAChR elongated oval plaque and topographical areas of the postsynaptic membrane become segregated into two well defined categories; some parts of the receptor pattern show organized folding, whereas other regions have more disorganized and less intensely reflecting receptor labeling.
- MS3. Oval nAChR plaque with one or more fluorescence-free ‘holes’. These holes are not innervated. As the holes enlarge, they connect to the outside of the junction by extended regions lacking receptor staining.
- MS4. Oval nAChR areas have been transformed into a more mature branched pattern, with a moderately convoluted external border and high- and low- receptor-density areas. The edge of the holes usually has a high receptor density.

- MS5. nAChR areas with a totally mature branched pattern with regions of high and very low or absent receptor density. The high-density regions are overlain by terminal branches and the other regions are not innervated.
- MS6. Discontinuous pattern of postsynaptic gutters resulting from the intercalary growth of the axon terminal.

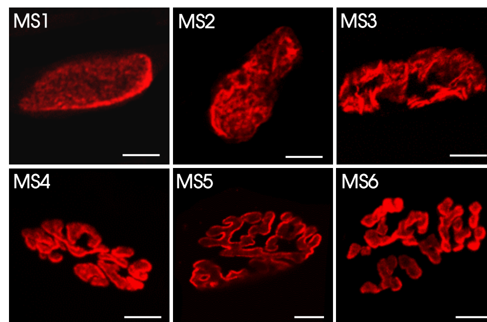


Figure 5. Synaptic nicotinic acetylcholine receptors (nAChR) cluster morphologies in the neuromuscular junctions of the mouse *Levator auris longus* (LAL) muscle from birth to adulthood. nAChRs were stained with rhodamine-conjugated α-bungarotoxin (α-BTX). Postsynaptic nAChR clusters are classified from MS1 to MS6 types according their morphologic maturation. Scale bar = 10 μm.

2.3. GLIAL COMPONENT

Schwann cells promote the growth and maintenance of the developing NMJ (Auld and Robitaille, 2003; Feng and Ko, 2008). SCs are shown to have two activities during synapse elimination: their processes separate nerve terminals from each other and from the muscle fiber; they contact the plaque of nAChR, apposing this surface as closely as the nerve, limiting the area where synaptic transmission occurs.

Terminal Schwann cells or teloglia, are present at the neonatal junction (Brill et al., 2011) and have been considered to play a role in synaptic competition (Smith et al., 2013). However, tSCs do play a major role in the consumption of the losing axons once they have been eliminated from the muscle surface (Bishop et al., 2004). As losing axons withdraw from the synapse, forming the previously mentioned retraction bulbs (Riley, 1977), tSCs phagocytose pieces of them (i.e., 'axosomes') (Bishop et al., 2004). tSCs also are important for the maintenance of axon terminals as their ablation in frog tadpoles reduces the growth of NMJs (Reddy et al., 2003). SCs deletion in mice (Wolpowitz et al., 2000) results in loss of muscle

innervation and MNs death. Interestingly, microglia in the CNS has been recently proposed to play a role in the postnatal elimination of retinal ganglion cell inputs to the lateral geniculate nucleus (Schafer et al., 2012) as well as synapse elimination in the hippocampus (Paolicelli et al., 2011).

Recently, it has been found that myelinating Schwann cells also have an important role in regulating synapse elimination at the mouse NMJ. In concrete, the loss of a single glial cell protein, the glial isoform of neurofascin (Nfasc155), was sufficient to disrupt postnatal remodeling of synaptic circuitry. Neuromuscular synapses were formed normally in mice lacking Nfasc155, including the establishment of robust neuromuscular synaptic transmission. However, loss of Nfasc155 was sufficient to cause a robust delay in postnatal synapse elimination at the NMJ (Roche et al., 2014).

2.4. MOLECULES INVOLVED IN SYNAPTIC ELIMINATION PROCESS

Several molecules contribute the development and stabilization of NMJs and are able to modify the function of the three cells of the NMJ (**figure 6**). Some of the most relevant ones are described below.

Neuregulin is a key molecule implicated in NMJ development. There are six closely related neuregulin genes, denoted Nrg1 to Nrg6 (Mei and Nave, 2014). Isoforms of Nrg1 and Nrg2 are expressed by MNs, MCs, and/or tSCs and are recruited to the postsynaptic muscle membrane (Moscoso et al., 1995; Rimer et al., 1997, 2004; Meier et al., 1998). Nrg1 in early postnatal development promotes plasticity of NMJs via its actions on tSCs (Lee et al., 2016). Specifically, during early stages, Nrg1 induce nAChR cluster density. Once nerve muscle contact is made, Nrg1 supports the differentiation of the pre- and postsynaptic components of the NMJ leading to the proper alignment. In late stage of NMJ development, Nrg1 promotes the engulfment of the NMJ by tSCs (Wang et al., 2017). Nrgs activate the receptor ErbB, which is a family of tyrosine kinase receptors comprising ErbB2, ErbB3, and ErbB4 isoforms, which are encoded by distinct genes. ErbB are stimulated by Nrgs through dimerization. Dimerization leads to auto- and transphosphorylation of tyrosine residues in their intracellular domains, which serve as docking sites for adaptor proteins and enzymes initiating downstream signaling cascades, including the Raf-MEK-ERK and JNK pathways. ErbB2, -3, and -4 are also expressed in the postsynaptic muscle membrane (Trinidad et al., 2000).

Another key molecule is agrin, a heparan sulfate proteoglycan synthesized and released by neurons, muscle fibers and Schwann cells (Ruegg et al., 1992). It is transported anterogradely to nerve terminals and released into synaptic clefts (Nitkin et al., 1987; Campagna et al., 1995). Neural agrin has been described as 1000-fold more effective in stimulating nAChR clustering than that synthesized by other cells (Reist et al., 1992; Zhang et al., 2016). Agrin can be considered both an inducer of nAChR clustering and a stabilizer of the postsynaptic membrane (Witzemann, 2006). An agrin coreceptor is the low-density lipoprotein receptor-related protein 4 (LRP4; Weatherbee et al., 2006; Kim et al., 2008; Zhang et al., 2008). LRP4 is concentrated at the NMJs and is necessary for agrin-stimulated muscle-specific tyrosine kinase receptor (MuSK) phosphorylation and nAChR clustering through the cytoplasmic linker protein rapsyn. Indeed, the knockout mouse models for either agrin or MuSK similarly cause dramatic reduction and alterations of nAChRs, leading to perinatal death (DeChiara et al., 1996; Gautam et al., 1996; Lin et al., 2001; Yang et al., 2001; Jing et al., 2009). MuSK is a single transmembrane tyrosine kinase receptor that is involved in all aspects of NMJ development (Hubbard and Gnanasambandan, 2013). As it has been described above, MuSK is the signaling component in the LRP4-MuSK receptor complex necessary for triggering postsynaptic differentiation upon binding to neural agrin (Kim et al., 2008; Ye and Rape, 2009). Moreover, it can, at least in part, induce a retrograde differentiation signal to the presynaptic motor axon (Kim and Burden, 2008).

Similar to the discovery of LRP4 being the coreceptor of MuSK, the identification of downstream of tyrosine kinases-7 (Dok-7) as an adapter necessary for the full activation of MuSK was based on the observation that mice deficient for Dok-7 die perinatally because of respiratory failure due to the lack of NMJs (Okada et al., 2006). A phosphorylation at the tyrosine residue of Dok-7 is needed for the binding of this to the cytoplasmic region of MuSK (Herbst and Burden, 2000; Herbst et al., 2002). Dok-7 belongs to a family of seven adaptor-like proteins (Dok-1 to Dok-7) that are characterized by NH2-terminal pleckstrin homology and phosphotyrosine-binding domains followed by Src homology 2 domain-containing (SHC) target motifs (Mashima et al., 2009). Several lines of evidence indicate that the Dok family members act as promiscuous cytosolic adapter molecules for tyrosine kinases. In some cases, binding of the Dok protein inhibits tyrosine kinase activation, whereas in others (as is the case for Dok-7), binding strongly enhances tyrosine kinase activity (Mashima et al., 2009).

Wnt proteins (from the portmanteau Wingless-related integration site) are secreted glycoproteins expressed as 15 isoforms in zebrafish and as 19 isoforms in mouse and humans. They are involved in multiple aspects of development (He et al., 2004; Van Amerongen and Nusse, 2009), including axon pathfinding and synaptogenesis. For instance, Wnts regulate nAChR clustering at vertebrate neuromuscular junctions (Luo et al., 2002; Wang et al., 2003) by interacting with MuSK or nAChR respectively (Jing et al., 2009; Zhang et al., 2012). Recently, it has been found that actin regulator cortactin levels increase at stimulated synaptic terminals and this increase requires neuronal activity, *de novo* transcription and depends on Wg/Wnt expression. Cortactin is necessary for the full range of activity-dependent plasticity, and probably it plays a direct role in the regulation of this process (Alicea et al., 2017).

Rapsyn is a cytoplasmic scaffolding protein expressed constitutively in myotubes; it is present at the NMJ from the earliest stages of development, and in adult, expression is largely restricted to the synaptic region. Rapsyn binds tightly to nAChRs to form a high-density network of the two proteins (Zuber and Unwin, 2013). Rapsyn also binds to dystroglycan (DG) (Bartoli et al., 2001) and is thus thought to link nAChRs to the postsynaptic actin cytoskeleton. The molecular signaling pathways that lead from MuSK activation to the clustering of the nAChR-rapsyn network are not well understood.

The structural specializations of the postsynaptic membrane involve, in addition to MuSK and rapsyn, the dystrophin-glycoprotein complex (DGC). The DGC is a transmembrane complex of proteins linking the actin cytoskeleton of the muscle fiber to the basal lamina. It includes (1) dystrophin or its homolog at the synapse, (2) utrophin, linked to cytoskeletal actin, (3) three groups of transmembrane proteins dystroglycan, (4) sarcoglycans and sarcospan, (5) and two groups of cytoplasmic proteins, the dystrobrevins and syntrophins (Singhal and Martin, 2011). In nonsynaptic muscle regions, the DGC is required to prevent muscle fiber damage caused by contraction, as shown in muscular dystrophies, which often result from mutations in components of the DGC (Davies and Nowak, 2006). Dystroglycan is posttranslationally cleaved into the transmembrane α-dystroglycan and the extracellular, sarcolemma-associated α-dystroglycan (Ibraghimov-Beskrovnaya et al., 1992). α-Dystroglycan is heavily glycosylated, and this glycosylation is essential for the binding to its ligands agrin, laminins, perlecan, neurexin, and pikachurin (Ervasti and Campbell, 1993; Bowe et al., 1994; Talts et al.,

1999; Sugita et al., 2001; Masiero et al., 2009). Accordingly, mutations of the enzymes that are involved in the glycosylation of these unusual sugar side chains cause underglycosylation of dystroglycan and can result in very severe, early-onset congenital muscular dystrophies (Moore and Winder, 2012).

Another protein involved in the development and stabilization of the NMJ is AChE. AChE is anchored in the extracellular matrix through the collagen protein ColQ (Krejci et al., 1997), which in turn forms complexes with different acceptor molecules like perlecan (Rotundo, 2003; Rotundo et al., 2005). This protein itself binds to dystroglycan to interact with laminin and agrin as well as with the cytoskeleton (Peng et al., 1998).

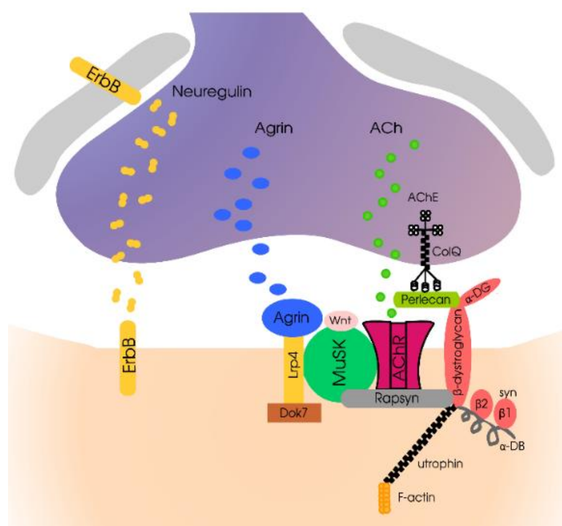


Figure 6. Some key molecules involved in neuromuscular junction development. Neuregulin is released and activates the receptor tyrosine kinases ErbB which are situated in the postsynaptic component and in Schwann cell. On the other hand, neural agrin binds to lipoprotein receptor-related protein 4 (LRP4). LRP4 is necessary for agrin-stimulated muscle-specific tyrosine kinase receptor (MuSK) phosphorylation. Downstream of tyrosine kinases-7 (Dok-7) is an adapter necessary for the full activation of MuSK. Several Wnts appear to act together with agrin to activate the LRP4-MuSK receptor. Acetylcholinesterase (AChE) is localized to the synaptic basal lamina and is essential to inactivate acetylcholine (ACh). The homotetrameric subunits encoded by the *Ache* gene, coassemble with the triple helical collagen tail, termed ColQ, which tethers the entire enzyme to synaptic basal lamina. The dystrophin-glycoprotein complex (DGC) contains dystroglycan (DG), which is posttranslationally cleaved into α-dystroglycan (αDG) and the transmembrane component β-dystroglycan (βDG). DG associates with rapsyn to link AChRs to the DGC and connects to α-dystrobrevin (αDB), to syntrophins (syn) and to utrophin. Utrophin links the entire complex to the F-actin cytoskeleton.

There are other proteins that are implicated in this process. Some of them have been extensively studied in our laboratory such as VDCCs, protein kinase C (PKC), mAChRs, NTRs, and ARs too (see extensively in section 3). All these molecules are related with ACh release in the nerve terminals during synaptic competition. It is known that the probability of ACh release is different during synaptic competition (Kopp et al., 2000). This neurotransmission switch could be preceded by withdrawal of the axons.

VDCCs (N, L and P/Q types) control the ACh release of the strong terminal ensuring the maximum entrance of Ca^{2+} and promoting their survival (Santafé et al., 2001, 2002). In contrast, in the weak terminal the entrance of Ca^{2+} by the same channels, activates a repressive mechanism of neurotransmission, which is controlled by PKC activity (Jia et al., 1999; Lanuza et al., 2002) causing the disconnection of the weak terminal (Santafé et al., 2001, 2002, 2007b). PKC involvement in the synaptic elimination process can be observed if their activity is blocked by Calphostin C (CaC). In concrete, CaC increases the number of polyinnervated axons, causing a delay in the synaptic elimination process when it is present (Lanuza et al., 2002).

mAChRs can directly modulate the synaptic efficacy of nerve terminals in polyinnervated NMJs during developmental synaptic elimination (Santafé et al., 2003, 2004). For example, weak endings were strongly potentiated by muscarinic 1 (M_1) receptor-selective antagonists (Santafé et al., 2009a). M_1 and muscarinic 4 (M_4) receptor subtypes of mAChRs are present in the weak nerve terminals and are individually coupled to inhibit ACh release (Santafé et al., 2004). Fully blocking all mAChRs in the weak ending with the unselective blocker atropine (the muscarinic 2 (M_2) receptor-subtype coupled to potentiation and the M_1- and M_4-subtype receptors both coupled to inhibition) potentiates release, which indicates that the end result of the compound mAChR-mediated modulation of ACh release in the weak synapses is to depress release strongly. Interestingly, the individual block of only M_1 or M_4 does not result in the recovery of silent synapses, although recovery was effective after all mAChRs had been blocked with atropine.

Neurotrophins and their receptors are also implicated in the synaptic elimination process. One example is the glial cell line–derived neurotrophic factor (GDNF). In transgenic mice that overexpress GDNF in muscle fibers, synapse elimination is delayed for several weeks (Nguyen et al., 1998), and in mice that are given large doses of GDNF daily by injection, the multiple innervation seems to

persist as long as the GDNF is given (Keller-Peck et al., 2001). Brain-derived neurotrophic factor (BDNF) is another well-known candidate involved in this process. *In vivo* (Je et al., 2013) and *in vitro* data (Yang et al., 2009; Je et al., 2012), suggest a new model in which a single molecule, BDNF, can be either the punishment or the reward signal, depending on proteolytic conversion. Thus, proBDNF from postsynaptic muscle cells serves as a 'punishment signal' that induces retraction of nerve terminals through $p75^{NTR}$. In parallel, neuronal activity drives secretion and/or activation of metalloproteases to convert proBDNF to mBDNF, a reward signal for which all terminals compete. In our laboratory, it has been studied the involvement of NTs in the synaptic elimination process. For example, the incubation of neurotrophin 3 (NT3) strongly potentiates evoked ACh release from the weak and the strong axonal inputs on dually innervated postnatal endplates (P6) but not in the most developed postnatal singly innervated synapses at P6. These results indicate that NT3 has a role in the developmental mechanism that eliminates redundant synapses though it cannot modulate synaptic transmission locally as the NMJ matures (Garcia et al., 2010c). Moreover, exogenously applied BDNF increases ACh release from singly and dually innervated synapses. This effect may be specific for BDNF because the neurotrophin 4 (NT4) (Garcia et al., 2010b) does not modulate release at P6-P7. Blocking the receptors TrkB and $p75^{NTR}$ completely abolishes the potentiating effect of exogenous BDNF (Garcia et al., 2011). These results suggest that a BDNF-induced $p75^{NTR}$-mediated ACh release potentiating mechanism and a BDNF-induced TrkB-mediated release inhibitory mechanism may contribute to developmental synapse disconnection (Garcia et al., 2010d). Finally, GDNF does not change the size of the evoked ACh release from the weak and the strong axonal inputs on dually innervated postnatal endplates nor in the most developed singly-innervated synapses at P6. These findings indicate that GDNF (unlike neurotrophins) does not acutely modulate transmitter release during the developmental process of synapse elimination (Garcia et al., 2010a).

■ 2.5. SYNAPTIC ACTIVITY

Activity-dependent synaptic competition plays a critical role in shaping patterns of synaptic connections in the nervous system. The most active terminal gets stabilized, whereas the less active ones withdraw (Benoit and Changeux, 1975; O'Brien et al., 1978; Nguyen and Lichtman, 1996). This drastic reduction in

polyneuronal innervation that occurs during the first postnatal weeks is produced by an activity-dependent competitive withdrawal of supernumerary axons (Benoit and Changeux, 1975; O'Brien et al., 1978; Jansen and Fladby, 1990; Ribchester and Barry, 1994).

The first hypothesis of the regulation of synapse elimination in relation with activity was that inactive synapses were permanently removed by the activity elicited by more active inputs innervating the same target cells. This is, in essence, the obverse of Hebb's well-known learning rule which enunciates that when an axon innervating a cell repeatedly or persistently does not take part in firing it, some growth process or metabolic change takes place on one or both cells, so that its efficiency as one of the cells firing the postsynaptic cell is decreased (Hebb, 1949). However, as with Hebb's rule, this mechanism maintains synchronously active inputs. Thus, active synapses prosper by punishing their inactive neighbors (Thompson, 1985). Axons may compete by generating activity-mediated signals to destabilize directly synaptic sites associated with other inputs. The withdrawal process is spatially regulated so the branches of an axon that are nearest (<50 μm) to the competitor's territory are removed before the more distant branches undergo retraction (Gan and Lichtman, 1998). This short distance is compatible with a local diffusion of ACh within the common synaptic gutter between competing endings. The different axonal inputs to a given NMJ in the *Levator auris longus* (LAL) muscle are generally intermingled (indeed, they share the same postsynaptic gutter of <10 μm) in the same endplate site, especially at the beginning and in the first half of the elimination process. However, although one neuron propagates the same activity pattern and level to all its axonal branches, the competitive success of these branches can vary greatly in different polyinnervated junctions (Keller-Peck et al., 2001).

Several studies have explored the effect of changes in neuromuscular activity on both the timing and the outcome of synapse elimination, either in development or during reinnervation of adult muscle (Ribchester, 1988; Favero et al., 2015). The activity is strongly influential but not strictly decisive in determining the outcome of the synapse elimination. Other variables likely to mitigate competitiveness include intrinsic limits on the numbers of peripheral connections any MN can support, sensitivity to neurotrophic factors, and selective recognition or adhesion of MN to specific muscle fibers based on either topographic or histochemical markers.

It is well established that the synaptic connections between motor axons and muscle are shaped by activity. Impaired postsynaptic activity at neuromuscular synapses delays the withdrawal of presynaptic terminals and synapse elimination (Bernstein and Lichtman, 1999). This has been demonstrated extensively for processes occurring late during synaptogenesis in which activity regulates synaptic maturation and refinement (Sanes and Lichtman, 1999). In the absence of activity, NMJs form an aberrant morphology with a reduction of postsynaptic specializations, as demonstrated in rat, *Drosophila melanogaster* and mouse (Duxson, 1982). The blockade of neural transmission leads to the loss of synapse elimination, causing aberrant branching of motor axons and multiple innervation of muscle fibers, altogether with modifications of MNs survival during normal cell death (Misgeld et al., 2002). It has been demonstrated that increasing activity accelerates the transition to monoinnervation. The synchronous activity of MNs first favors polyneuronal innervation, whereas asynchronous activity subsequently promotes synapse elimination (Buffelli et al., 2002). Interestingly, the blockade of AP generation in muscle can inhibit synapse elimination through local signaling (Favero et al., 2009). Moreover, activity-dependent mechanism based on timing, seems to assure that at the end of the competitive process all polyneuronal innervation is eliminated and a single motor terminal invariably remains on each myofiber (Favero et al., 2012).

Adaptive changes in the output of neural circuits underlying rhythmic behaviors are relayed to muscles via motor neuron activity. Pre- and postsynaptic properties of neuromuscular junctions can impact the transformation from motor neuron activity to muscle response. Further, synaptic plasticity occurring on the time scale of inter-spike intervals can differ between multiple muscles innervated by the same motor neuron. In rhythmic behaviors, MN bursts can elicit additional synaptic plasticity. However, it is unknown if plasticity regulated by the longer time scale of inter-burst intervals also differs between synapses from the same neuron, and whether any such distinctions occur across a physiological activity range (Blitz et al., 2017).

3. MUSCARINIC, ADENOSINE AND NEUROTROPHIN RECEPTORS IN SYNAPTIC ELIMINATION PROCESS

3.1. MUSCARINIC RECEPTORS

ACh activates two structurally and functionally distinct types of receptors: nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). nAChRs function as ionotropic receptors, whereas mAChRs are metabotropic receptors signaling through G-proteins. The five mammalian mAChR subtypes, M_1–M_5, comprise two functionally distinct groups: odd-numbered mAChRs (M_1, M_3, M_5) preferentially activate G_q/G_{11}-type G-proteins, while even-numbered mAChRs (M_2, M_4) activate G_i/G_0-type G-proteins (Kurowski et al., 2015).

Through their linkage to G_q/G_{11}, M_1, M_3 and M_5 receptors predominantly activate phospholipase C (PLCγ) via the α-subunit (**figure 7**). The activation of PLCγ results in production of diacylglycerol (DAG), which is generated together with inositol trisphosphate (IP_3) upon the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PLCγ. This path facilitates the mobilization of intracellular Ca^{2+}, activate the PKC and subsequently, mitogen-activated protein kinase (MAPK). On the other hand, M_2 and M_4 receptors mainly inhibit the adenylyl cyclase (AC) through their corresponding G proteins, leading to a decrease in cyclic adenosine monophosphate (cAMP) levels. The reduction of cAMP production results in a decrease in the activity of protein kinase A (PKA).

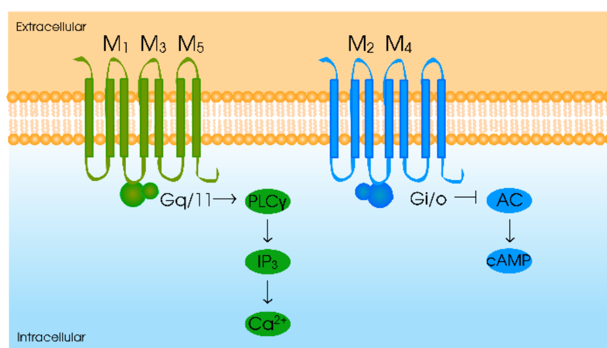


Figure 7. Muscarinic acetylcholine receptors (mAChR) can be subdivided based upon their G-protein-coupling characteristics and effector mechanisms. M_1, M_3 and M_5 mAChRs preferentially couple to G-proteins of the G_q/G_{11} family, whereas M_2 and M_4 receptors activate G-proteins of the G_i/G_0 family. M_1, M_3 and M_5 receptors stimulate phospholipase C (PLCγ) which increase inositol trisphosphate (IP_3) and facilitate the mobilization of intracellular Ca^{2+}. M_2 and M_4 receptors inhibit adenylyl cyclase (AC) and, therefore, decrease the cyclic adenosine monophosphate (cAMP) levels.

Most tissues and cell types express two or more mAChR subtypes that exert diverse physiological actions, depending on the cellular location and identity of the receptor subtypes (Wess et al., 2007; Nathanson, 2008). In the nervous system, mAChRs act primarily as modulators of synaptic transmission, regulating cognitive, sensory, motor, and autonomic functions, and are implicated in the pathophysiology of illnesses such as Alzheimer's disease, Parkinson's disease, depression, and schizophrenia (Wess, 2004; Langmead et al., 2008). It remains unknown, however, whether muscarinic signaling plays a structural role at the synapse.

3.1.1. mAChRS IN THE NMJ

The role of presynaptic mAChRs is well-documented in the CNS but is less clear at the NMJ. mAChRs exhibit both facilitating and inhibitory actions on transmitter release, based on the effects of selective agonists and antagonists (Van der Kloot and Molgó, 1994). All mAChRs subtypes are present in rat and mouse skeletal NMJ in both newborn and adult stages (Garcia et al., 2005) and the presence of these receptor subtypes in the mammalian motor endplates has been functionally tested (Santafé et al., 2003, 2004). Moreover, it is known that mAChRs are involved in both the inhibition and enhancement of ACh release in cholinergic synapses (Ganguly and Das, 1979; Abbs and Joseph, 1981; Wessler et al., 1987; Arenson, 1989; Caulfield MP, 1993; Caulfield and Birdsall, 1998; Nathanson, 2000; Parnas et al., 2000). However, the subtypes of mAChRs related to the modulation of neurotransmitter release in the various central and peripheral cholinergic circuits during development and in the adult are not fully resolved (Allen et al., 1999; Arellano et al., 1999; Slutsky et al., 1999; Minic et al., 2002; Santafé et al., 2003, 2004). A dual M_1/M_2 (enhancement/inhibition) pattern of mAChRs functional expression has been found at the adult skeletal NMJs (Slutsky et al., 1999; Minic et al., 2002; Santafé et al., 2003). It has also been observed that M_1, M_2 and M_4 muscarinic autoreceptors are differentially expressed in nerve endings on the dually- and monoinnervated synapses of the newborn mammals. In concrete, these receptors are differentially linked to the L-, N- and P/Q-type VDCC, and may directly modulate both synaptic consolidation (transmitter release potentiation in the most active ending) and elimination (depression in certain less active endings) during axonal competition and neural connectivity maturation (Santafé et al., 2004).

Our group found that M_3 receptors are not coupled to release modulation in neither newborn nor adult NMJs. However, M_3 has been found previously in Western blots from innervated areas of the mouse diaphragm (Minic et al., 2002).

3.1.2. mAChRS AND SYNAPSE ELIMINATION

There are some evidences which show that mAChRs are involved in the synaptic elimination process. Previous studies by our group and others observed that presynaptic mAChRs can directly modulate the synaptic efficacy of nerve terminals in polyinnervated NMJs during developmental synaptic elimination (Santafé et al., 2003, 2004, 2007b, 2009a, 2009b; Tomàs et al., 2011). Specifically, it was found that in polyinnervated synapses, the general action of all selective M_1 and M_2 blockers tested is to reduce the release. Noteworthy, this mAChR action is different for each terminal of dual junctions still in competition. In concrete, both M_1 and M_2 blockers reduce the release in the strongest ending but in the weakest nerve terminal in dual junctions, only M2 blockers reduce release.

Moreover, it was observed a graded change from a multichannel involvement (P/Q- N- and L-type voltage-dependent calcium channels) of all muscarinic responses (M_1-, M_2- and M_4-mediated) in the small-EPP to the single channel (P/Q-type) involvement of the M_1 and M_2 responses in the singly innervated endplates. This indicates that muscarinic autoreceptors can directly modulate large-EPP generating ending potentiation, and small-EPP generating ending depression through their association with the calcium channels during development. Thus, the involvement of presynaptic mAChRs in the elimination process may allow direct interaction between nerve endings through differential activity-dependent ACh release (Santafé et al., 2009a).

3.1.3. MUSCARINIC AGONISTS AND ANTAGONISTS

3.1.3.1. Muscarinic agonists

All muscarinic agonists are similar to acetylcholine in structure. Muscarinic agonists contain the positively charged quaternary ammonium group and a partially negatively charged ester group. Most of the agonists bind the receptors at the orthosteric site, i.e. the same site of ACh binding (Gregory et al., 2007). The site of agonist binding is a circular pocket formed by the upper portions of the

seven membrane-spanning regions. Muscarinic agonists decrease the heart rate, cause vasodilation (Harvey, 2012), smooth muscle contraction, lacrimation, sweating, salivation, bronchial secretion and contraction of ciliary muscle (Rang et al., 2007; Goodman et al., 2011; Clark et al., 2012). M_1-mAChR agonists lack specificity and have low bioavailability, characteristics that reduce their therapeutic window and limit their clinical uses as agonists. However, the muscarinic agonist pilocarpine is used clinically to treat glaucoma (Fisher et al., 2002; Rang et al., 2007; Goodman et al., 2011; Clark et al., 2012). Of the muscarinic agonists, oxotremorine M is the most potent muscarinic agonist.

Oxotremorine M

Oxotremorine is a potent centrally and peripherally acting muscarinic cholinergic agonist (Bebbington et al., 1966), which has been shown to be active in isolated tissue preparations as well as *in vivo* (Ringdahl and Jenden, 1983).

Moreover, oxotremorine M (oxo-M) a quaternary nitrogen analog of oxotremorine, is a full agonist for the phosphatidyl-inositol response, while oxotremorine is a partial agonist (Fisher et al., 1984). Both oxotremorine and oxo-M are full agonists for inhibition of adenylate cyclase (Olianas et al., 1983; Brown and Brown, 1984).

3.1.3.2. Muscarinic antagonists

Muscarinic antagonists contain the quaternary/tertiary ammonium group with a big aromatic group and an ester group. They are lipid soluble. Muscarinic antagonists bind to the orthosteric site of the receptors to block the active site of receptor. Their effect on heart causes tachycardia, stops the secretions like saliva, tears, dilation of pupil, bronchial dilation and, on central nervous system, excitation. The muscarinic antagonists are clinically used to treat peptic ulcer, asthma, bronchitis, urinary incontinence (Rang et al., 2007; Goodman et al., 2011; Clark et al., 2012). Classical muscarinic antagonists include atropine, tiotropium, pirenzipine, darifenacine, ipratropium bromide and scopolamine (Martos et al., 1987; Rang et al., 2007; Goodman et al., 2011; Baysinger et al., 2012). M_1-mAChRs have high affinity for pirenzepine and also for biperiden (Klinkenberg et al., 2013). Gallamine is suggested to decrease cardiac rate and force of contraction because it blocks M_2-mAChRs (Dai et al., 2012). Other selective muscarinic antagonists for this

receptor include tripitramine (Gamberini et al., 2012) and methoctramine (Angeli et al., 1995; Giglio et al., 2007).

In our laboratory, mAChRs antagonists have been extensively used for many studies. For instance, to evaluate whether mAChRs can directly modulate the synaptic efficacy of nerve terminals in polyinnervated NMJs during developmental synaptic elimination (Santafé et al., 2003, 2004). Additionally, antagonists of these receptors were used to study the action of mAChRs on PKC activity in the adult NMJ (Obis et al., 2015b).

Atropine

Atropine (AT) is a tropane alkaloid extracted from *Atropa belladonna*. AT is a non-selective muscarinic receptor antagonist and blocks the acetylcholine vascular relaxation selectively. Anticholinergics have potential psychoactive properties. For instance, AT affects the synthesis of serotonin in the serotonergic pathways (Kumari et al., 2007). AT is an important tool to understand the neurobiology of the cholinergic system, because of its anticholinergic property at postganglionic parasympathetic muscarinic sites. Muscarinic receptors antagonists are important tools for physiological, behavioural and neurological studies and the characterization of AT and other anticholinergics are very important to improve research studies.

Pirenzepine

Pirenzepine (PIR) is a selective antagonist for the M_1-mAChR and is reported to block presynaptic M_1-mAChR as well as some reported inverse agonist activity. Pirenzepine displays higher affinity for M_1 than M_2 receptors. This inhibitor is used to treat gastric ulcers, as it reduces gastric acid secretion and muscle spasms.

Methoctramine

Methoctramine (MET) is a selective antagonist of M_2-mAChR (Wess et al., 1988) which interacts with both the orthosteric and the allosteric binding sites located between the second and third extracellular loops (Jakubík and El-Fakahany, 2010). It shows allosteric properties at high concentrations. However, methoctramine may also act as an antagonist on nicotinic receptors in the airways (Watson et al., 1992). This blocker is the major tool for the functional studies on muscarinic receptor subtype M_2 because of its relative specificity. It may, however, show some cytotoxicity effects at high concentrations (Zini et al., 2009).

Muscarinic toxin 3

Muscarinic toxin 3 (MT3) was isolated from the venom of the African snake *Dendroaspis angusticeps* (green mamba) and its amino acid sequence has been determined. Its ability to inhibit the binding of (3H)N-methylscopolamine ((3H)NMS) to Chinese hamster ovary cells stably expressing subtypes of muscarinic receptors was studied. MT3 displayed high affinity for the M_4-mAChR (pKi = 8.7 ± 0.06), 40-fold lower affinity at M_1-mAChR (pKi = 7.11 ± 0.17) whereas no inhibition of (3H)NMS binding to M_2,M_3,M_5-mAChR, was observed at concentrations up to 1 μM. This makes MT3 the most selective M_4-mAChR ligand known to date (Jolkkonen et al., 1994).

3.2. ADENOSINE RECEPTORS

Activation of adenosine receptors (ARs), also known as a P1 purinergic receptors or P_1Rs, is dependent on the presence of extracellular adenosine which can bind to a family of four receptors, termed adenosine A_1 receptor (A_1), adenosine A_{2A} receptor (A_{2A}), adenosine A_{2B} receptor (A_{2B}), and adenosine A_3 receptor (A_3). All four receptors are G-protein coupled, either G_s or G_i and signal primarily through the activation (A_{2A} and A_{2B}) or inhibition (A_1 and A_3) of cAMP (**figure 8**). The four receptors have also been reported to activate PLCγ and MAPK (Fredholm et al., 2001). There is strong homology between specific ARs in different species and all are asparagine-linked glycoproteins with seven transmembrane sequences. The A_1 receptor comprises six exons (two of which are coding) and the other ARs have two coding exons (Olah and Stiles, 2000; Fredholm et al., 2001; Haskó et al., 2005). A_1 and A_{2A}, in particular, can heterodimerize with D_1 and D_2 dopamine receptors (Kudlacek et al., 2003; Fuxe et al., 2007; Kim and Palmiter, 2008) and Group 1 and 2 metabotropic glutamate receptors (Ferré et al., 1999) providing another level of functional control. There is also evidence to suggest that adenosine can interact or activate ion channels such as L- and N-type calcium channels (Mei et al., 1996), VDCC (Chieng and Bekkers, 2001; McCool and Farroni, 2001) and K^+ channels (Paisansathan et al., 2010). ARs are widely expressed, and have different affinities for adenosine – A_1 and A_{2A} are to a certain extent high affinity receptors (K_m< 30 nM) whereas the A_3 and particularly the A_{2B} receptor are low affinity receptors (K_m 1–20 μM). The A_{2B} and A_3 receptors are thus only likely to be activated under high metabolic and stressful cellular conditions (Fredholm et al., 2001; Pedata et al., 2007).

In terms of structure, adenosine receptors are polypeptides of 36-45 kDa with an extracellular N-terminal and an intracellular C-terminal and seven transmembrane segments which are connected by three extracellular and three cytoplasmic hydrophilic loops (Ralevic and Burnstock, 1998; Fredholm et al., 2001; Burnstock, 2007). The N-terminal domain has N-glucosilation sites while the C-terminal domain contain serine and threonine residues, which are phosphorylation sites for protein kinases and allow the desensitization (Baldwin et al., 1994). The ligand binding to the receptor, takes place in transmembrane segments in the extracellular domain. ARs differ in their length and the function of their extracellular N-terminal domain, their intracellular C-terminal domain and the loops intra-, extracellular (De Lera Ruiz et al., 2014).

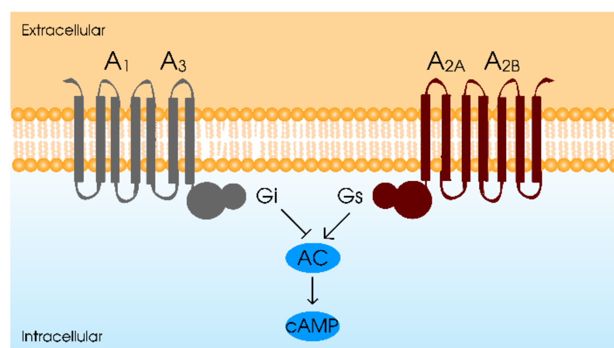


Figure 8. Classification of adenosine receptors (AR) according its signaling pathway. Adenosine mediates its action via four G-protein coupled receptors, A_1, A_{2A}, A_{2B} and A_3 that are coupled primarily to the activation and inhibition of cyclic adenosine monophosphate (cAMP).

Moreover, adenosine receptors are implicated in different diseases such as Parkinson, inflammation and ischemia (Federico and Spalluto, 2012; Villar-Menéndez et al., 2014). A_{2A} has emerged as an attractive non-dopaminergic target in the pursuit of improved therapy for Parkinson's disease, based in part on its unique CNS distribution. It is highly enriched in striatopallidal neurons and can form functional heteromeric complexes with other G-protein-coupled receptors. Blockade of the adenosine A_{2A} in striatopallidal neurons reduces postsynaptic effects of dopamine depletion, and in turn lessens the motor deficits of Parkinson disease (Schwarzschild et al., 2006).

3.2.1. ARs IN THE NMJ

Since the pioneering studies of Ribeiro and Walker, 1975, it is now known that adenosine and ATP released by nerve endings modulate the presynaptic metabolism through purinergic autoreceptors (Correia-de-Sá et al., 1991). It has been reported that micromolar concentrations of the AR endogenous agonist adenosine reduced evoked quantal content and/or spontaneous ACh release in frog neuromuscular junctions (Searl and Silinsky, 2005; Shakiryanova et al., 2006; Adámek et al., 2010) and rat (De Lorenzo et al., 2006; Pousinha et al., 2010). In other studies in mice, only very high adenosine concentrations reduce perineural calcium currents and affect neurotransmission (Silinsky, 2004).

Our group confirmed that the four subtypes of adenosine receptors are present in the motor endings. A_1 localizes in the terminal Schwann cell and nerve terminal, whereas A_{2A} localizes in the postsynaptic muscle and in the nerve terminal (Garcia et al., 2013). A_1 is more abundant in the adult whereas A_{2A} is more abundant in the newborn. This suggests some differential involvement of A_{2A} in the postnatal synapse elimination process. Furthermore, A_{2B} and A_3 receptors are present in the nerve terminal and muscle cells at the NMJs. Neither A_{2B} nor A_3 receptors are localized in the Schwann cells (Garcia et al., 2014).

The presence of these receptors in the neuromuscular synapse allows the receptors to be involved in the modulation of transmitter release. The role of the AR can conserve resources by limiting spontaneous quantal leak of ACh and protect the synapse function by decreasing the magnitude of depression during synaptic activity (Garcia et al., 2013).

3.2.2. ARs AND SYNAPSE ELIMINATION

Since adenosine is an endogenous purine with an important role in the regulation of neuronal excitability and low-frequency synaptic transmission, adenosine could modulate phenomena of synaptic plasticity. Through A_1 and A_{2A} receptor activation, adenosine modulates neuronal homeostasis and tunes the ability of synapses to undergo and/or sustain plasticity (Dias et al., 2013).

There are several findings that involve adenosine receptors as important molecules in synaptic plasticity. However, no information on the role of these receptors in the synaptic elimination process is published. It is known that selective

A_1 antagonists facilitate, whereas selective adenosine A_{2A} receptor antagonists attenuate, long term potentiation (LTP) in the hippocampus. Although caffeine is a non-selective antagonist of adenosine receptors, it attenuates frequency-induced LTP in hippocampal slices in a manner similar to selective adenosine A_{2A} receptor antagonists (Costenla et al., 2010). Also it was found that the selective A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) could increase the magnitude of long-term depression (LTD) normally elicited in neonatal rats (De Mendonça et al., 1997) and allow the emergence of LTD in young adult rats that usually do not express it (Kemp and Bashir, 1997). This suggest a tonic inhibitory effect of endogenous adenosine, acting through A_1 receptors, on LTD.

Recently, it has been found that ATP may activate neuronal P2Y1 receptors to promote synapse elimination (Yang et al., 2016). This hypothesis is supported by a previous study showing that activation of neuronal P2Y1 receptors is required for astrocytes derived ATP mediated LTD in hippocampus (Chen et al., 2013). Due to a close linkage proposed between LTD and synapse elimination (Wiegert and Oertner, 2013), one possible model is that ATP and downstream purinergic signaling could recognize unwanted synapses. Both this findings and the localization of the adenosine receptors makes them a great candidates in the involvement of synaptic elimination process.

3.2.3. ADENOSINE AGONISTS AND ANTAGONISTS

3.2.3.1. Adenosine agonists

Although adenosine is the endogenous agonist of the adenosine receptors, it is metabolically unstable, subjected to absorption by glial and neuronal cells, and to enzymatic inactivation because it can be the substrate of several enzymes (Wu and Phillis, 1982). The use of selective agonists, being more stable than the natural ligand (adenosine) has facilitated the functional study of these receptors (Fredholm et al., 2001). However, the structural basis of all known ARs agonists is related to adenosine with some small changes (at the position 5 of ribose, the position N6- and C2- of purine adenine ring) to increase the affinity for receptor subtypes without destroying the activity of the agonist (Burnstock, 2007; Della Latta et al., 2013). One example of unselective agonist used in this thesis for synapse elimination studies is adenosine 5′-triphosphate disodium salt hydrate.

Adenosine

Adenosine 5′-triphosphate disodium salt hydrate (Adenosine, ADO) is a central component of energy storage and metabolism *in vivo*. ATP is used in many cellular processes like respiration, biosynthetic reactions, motility, and cell division. ATP is a substrate of many kinases involved in cell signaling and of adenylate cyclase that produce the second messenger cAMP. ATP provides the metabolic energy to drive metabolic pumps. ATP serves as a coenzyme in a wide array of enzymatic reactions.

In general, adenosine has an inhibitory effect in the central nervous system. Caffeine's stimulatory effects are credited primarily (although not entirely) to its capacity to block adenosine receptors, thereby reducing the inhibitory tonus of adenosine in the CNS. This reduction in adenosine activity leads to increased activity of the neurotransmitters dopamine and glutamate. Experimental evidence suggests that adenosine and adenosine agonists can activate Trk receptor phosphorylation through a mechanism that requires the A_{2A} receptor (Lee and Chao, 2001).

The endogenous agonist adenosine is being used in hospitals as treatment for severe tachycardia, (Peart and Headrick, 2007) and acting directly to slow the heart through action on all four adenosine receptors in heart tissue (Cohen and Downey, 2008) as well as producing a sedative effect through action on A_1 and A_{2A} receptors in the brain.

Moreover, in our laboratory has been extensively used to study the function of adenosine receptors in the neuromuscular junction. For instance, to investigate the relation between adenosine receptors pathway and PKC signaling (Obis et al., 2015b) and to know the relation between adenosine and muscarinic receptors in acetylcholine release modulation (Santafé et al., 2015).

3.2.3.2. Adenosine antagonists

Adenosine receptor antagonists can be divided into two groups: xanthines and derivatives of xanthines and the non-xanthines. Most antagonists are derived from xanthines, which are based on the introduction of a hydrophobic substituent (phenyl or cicloalquil) at position 8 of the xanthine ring (Fredholm et al., 2001; Della Latta et al., 2013). Examples of the derivatives of xanthines are caffeine and theophylline, which are no-specific antagonists of natural adenosine (Della

Latta et al., 2013). The triazoloquinazoline CGS 15943 has been used for some years as a selective A_{2A} receptor antagonist. However, the compound also has high affinity for A_1, A_{2B}, and human A_3 receptors. An important step forward has been made with the discovery that xanthines, upon appropriate chemical manipulation, can become A_{2A} receptor antagonists (Baraldi et al., 2004). These compounds have, however, some limitations which prevent their wide use as pharmacological tools. For example, they undergo rapid photoisomerization, their affinity and selectivity are good but not very high, and the only radioligand available, (3H)-KF17837, has an elevated nonspecific binding (Ongini and Fredholm, 1996). Nevertheless, these compounds are of great interest in their potential for treatment of Parkinson's disease (Richardson et al., 1997). Specifically, for the study of synaptic elimination process of this thesis, it has been used a xanthine derivative called DPCPX (A_1 receptor) and non-xanthine derivative called SCH-58261 (A_{2A} receptor). These antagonists also were used to study the implication of ARs in the modulation of ACh release (Garcia et al., 2013; Santafé et al., 2015).

8-SPT

8-(p-sulfophenyl)theophylline (8-SPT) is an adenosine receptor antagonist. A sulfonate group was introduced at the *p*-position of the 8-phenyl ring, which greatly increased the water solubility (Daly et al., 1985; Shamim et al., 1989). Thus, 8-SPT is useful in pharmacological experiments where a blockade of all AR subtypes is required. It has to be kept in mind that these compounds do not block rat A_3 receptors but are active at other species like human and sheep. 8-SPT was shown not to penetrate into the brain due to its high polarity (Baumgold et al., 1992).

DPCPX

8-cyclopentyl-1,3-dipropylxanthine (DPCPX) competitively antagonized both the inhibition of adenylate cyclase activity via A_1 receptors and the stimulation via A_2 receptors (Lohse et al., 1987; Martinson et al., 1987). It has high selectivity for A_1 over other adenosine receptor subtypes, but as with other xanthine derivatives DPCPX also acts as a phosphodiesterase inhibitor, and is almost as potent as rolipram at inhibiting phosphodiesterase-4 (Ukena et al., 1993). It has been used to study the function of the A_1 receptor in animals, (Coates et al., 1994; Moro et al., 2006) which has been found to be involved in several important functions such as regulation of breathing (VanDam et al., 2008) and activity in various regions of the brain (Migita et al., 2008; Wu et al., 2009). DPCPX has also been shown to produce behavioural effects such as increasing the hallucinogen-

appropriate responding produced by the serotonin 2A receptor (Marek, 2009) and the dopamine release induced by 3-4-methylenedioxymethamphetamine, (Vanattou-Saïfoudine et al., 2011) as well as having interactions with a range of anticonvulsant drugs (De Sarro et al., 1996; Chwalczuk et al., 2008).

SCH-58261

2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo(4,3-e)(1,2,4)triazolo(1,5-c)pyrimidin-5-amine (SCH-58261) is a highly potent and selective antagonist of A_{2A} receptor. SCH-58261 has emerged as a very interesting tool. The compound has high affinity and is selective for A_{2A} receptors located on a variety of cell types from different animal species, including humans. SCH-58261 behaves as a competitive A_{2A} receptor antagonist and is effective *in vivo*, where it blocks the effects of A_{2A} receptor agonists. Ongoing efforts will allow researchers to better understand the specific function of A_{2A} receptor in physiological or altered conditions. Blockade of A_{2A} receptor is also of interest for drug development as 8-styrylxanthines and SCH-58261 have been found to be effective in animal models of Parkinson's disease and stroke (Monopoli et al., 1998; El Yacoubi et al., 2001).

■ 3.3. NEUROTROPHIN RECEPTORS

Neurotrophins (NT) and their receptors (NTR) were identified as promoters of neuronal survival, but they also regulate many aspects of neuronal development and function, including synapse formation and synaptic plasticity (Bibel and Barde, 2000; Kaplan and Miller, 2000; Huang and Reichardt, 2001, 2003; Poo, 2001; Sofroniew et al., 2001; Dechant and Barde, 2002; Chao, 2003).

There are different neurotrophins and neurotrophin receptors. One of the most well-known receptor is tropomyosin receptor kinase (Trk), a family of three receptor tyrosine kinases (TrkA, TrkB and TrkC), each of which can be activated by one or more of four neurotrophins —nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4/5 (NT3 and NT4/5). The other receptor is $p75^{NTR}$ which is able to bind all neurotrophins with the same affinity.

Specifically, TrkB receptor binds the neurotrophins BDNF as well as NT3 and NT4/5 and has three main parts: extracellular, transmembrane and intracellular domains (**figure 9**). At the extracellular side, they have a cysteine-rich cluster, three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin-like domains (binding sites for BDNF and NT4). The transmembrane region passes the

membrane once and is followed by the cytoplasmic tyrosine kinase domain (Segal, 2003).

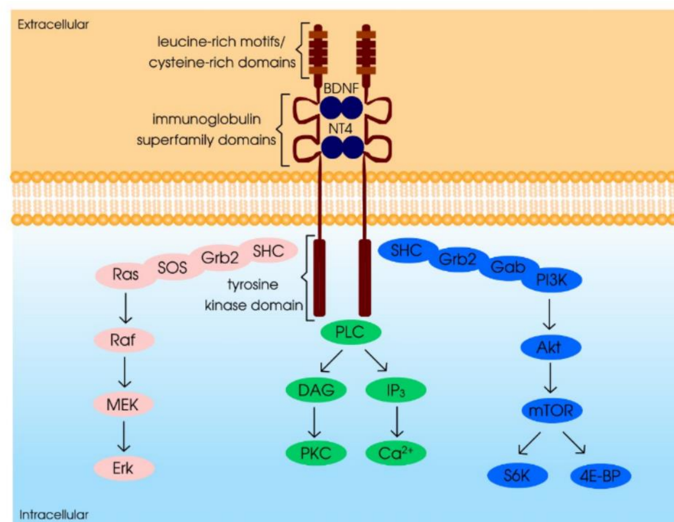


Figure 9. Tropomyosin-related kinase B (TrkB) receptor signaling pathway. TrkB receptor consists of two cysteine-rich domains, a cluster of three leucine-rich motifs, and two immunoglobulin-like domains in the extracellular moiety. Upon binding to brain-derived neurotrophic factor (BDNF) or neurotrophin 4 (NT4), a series of tyrosine phosphorylation events occur within TrkB tyrosine kinase domain. These phosphotyrosine residues form unique binding sites for intracellular adaptor proteins, Y516 and Y817 recruiting specifically Src homology 2 domain-containing (SHC) and phospholipase C (PLCγ), respectively. The typical wave of second messengers involves, PLCγ, which raises intracellular calcium and diacylglycerol (DAG).

Activation of TrkB upon BDNF binding leads to receptor dimerization and phosphorylation, thereby creating docking sites for effector proteins that initiate the activation of intracellular signaling pathways (Cunha et al., 2010). The phosphorylated tyrosine residues Y516 and Y817 in human TrkB receptor serve as the main docking sites to initiate downstream signaling pathways, such as Src homology 2 domain-containing (SHC), Akt, mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (Erk) 1/2 and PLCγ (**figure 9**) (Poo, 2001; Chao, 2003; Huang and Reichardt, 2003; Arevalo et al., 2006; Woo and Lu, 2006; Castrén and Rantamäki, 2010; Cunha et al., 2010). This in turn leads to the activation of various pathways involved in cellular functions that range from initiation of gene transcription and protein synthesis to decisions involved in cell growth and survival. The residues Y702, Y706 and Y707, located within the tyrosine

kinase domain, can also recruit adaptor proteins when phosphorylated, including Grb2 and SH2B. At the same time as inducing multiple protein tyrosine, serine and threonine phosphorylation events in diverse proteins, BDNF stimulation may cause a reduction in the phosphorylation of other proteins, such as focal adhesion kinase (Spellman et al., 2008), thereby reducing their activity.

3.3.1. TrkB IN THE NMJ

Activation of the TrkB by its ligands, BDNF, NT3 and NT4/5, is decisively important for the development, survival and plasticity of the nervous system (Bibel and Barde, 2000; Huang and Reichardt, 2003; Lu, 2003).

TrkB is expressed in muscle and colocalizes with postsynaptic nAChRs and with the nerve terminal and Schwann cell (Gonzalez et al., 1999; Garcia et al., 2010e). Accordingly, both BDNF and NT4 have been shown to be expressed in MN cell bodies and axons (Pitts et al., 2006; Garcia et al., 2010f) in the muscle targets (Oppenheim et al., 1992; Sendtner et al., 1992; Funakoshi et al., 1993; Garcia et al., 2010f) and in Schwann cells (Garcia et al., 2010f; Wilhelm et al., 2012).

Signaling via TrkB receptors modulates neurotransmitter release by interacting with presynaptic muscarinic receptors (Garcia et al., 2010e). In addition, BDNF-induced TrkB signaling contributes to the stabilization of polyinnervated NMJs during the postnatal period of synaptic elimination (Garcia et al., 2010d).

Genetic knockdown models lacking TrkB receptors or BDNF die during embryonic or early postnatal development (Snider, 1994), and heterozygous models with reduced expression do not avoid possible developmental effects on NMJ function and structure. This means that other receptors are involved in postnatal development.

3.3.2. TrkB AND SYNAPSE ELIMINATION

Neurotrophins also modulate the synaptic activity of NMJs (Wang and Poo, 1997). In fact, electrical activity regulates the synthesis (Gall and Isackson, 1989; Funakoshi et al., 1995) and secretion (Blöchl and Thoenen, 1995; Wang and Poo, 1997) of neurotrophins and the expression of their receptors during development and thus modulate synapse elimination.

In a previous study, our group found that exogenously applied BDNF during development (10 nM for 3h) also increases ACh release from singly and dually innervated synapses at P6–P7 with the involvement of TrkB and $p75^{NTR}$ receptors (Garcia et al., 2010d). This effect may be specific to BDNF because NT4 does not modulate release at P6–P7 (Garcia et al., 2010b). However, it has been reported that low doses of BDNF rapidly induce (within minutes) a TrkB-dependent potentiation of both spontaneous and evoked synaptic activity at developing neuromuscular junctions in *Xenopus laevis* studied in culture (Stoop and Poo, 1996; Poo and Boulanger, 1999; Poo, 2001). In addition to possible species difference, the lack of a short-term effect of neurotrophins on static EPP amplitude in our NMJ model may misrepresent dynamic neuroplasticity effects. Another study shows that exogenous application of mBDNF triggers synaptic potentiation and maturation of developing NMJs through TrkB, whereas application of proBDNF suppresses synaptic transmission and causes axonal retraction by activating presynaptic $p75^{NTR}$ (Yang et al., 2009).

Moreover, the blockade of $p75^{NTR}$ signaling attenuated synapse elimination, whereas the blockade of TrkB signaling, or inhibition of proBDNF cleavage by metalloproteases, promoted synaptic retraction of both innervated axon terminals in triplets. Taken together, these findings suggest a model for synapse elimination in which the activity-dependent conversion of proBDNF to mBDNF selectively stabilized active terminals, whereas inactive terminals were eliminated in response to proBDNF and subsequent activation of $p75^{NTR}$ signaling (Je et al., 2012).

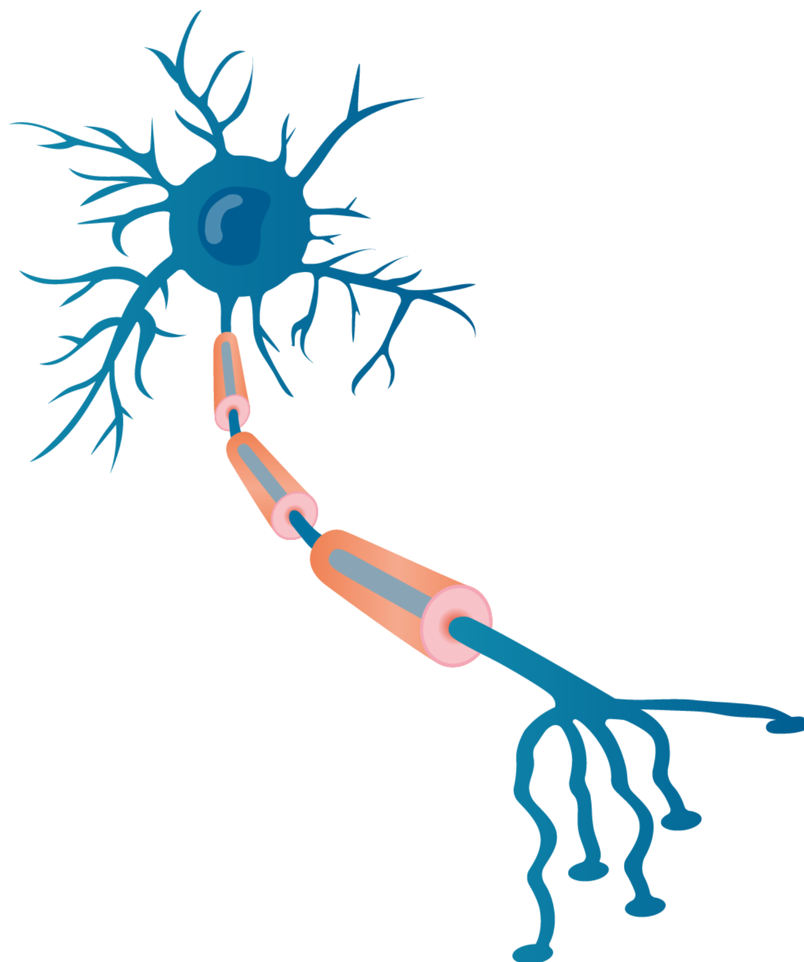
3.3.3. TrkB ANTAGONIST

TrkB-Fc chimera

TrkB-Fc chimera is a fusion protein combining the extracellular binding domain of TrkB and the histidine-tagged Fc region of human IgG in order to block TrkB ligand signaling. TrkB-Fc is a tool for studying the biological actions of BDNF.

A large number of *in vitro* studies support the notion that TrkB-Fc inhibits BDNF activity (Shelton et al., 1995; Mantilla et al., 2013; Obis et al., 2015a). Addition of TrkB-Fc to hippocampal and cortical slices and cultured cortical, striatal, and dentate granule cells either abolishes or opposes the effect of BDNF. In addition, administration of TrkB-Fc *in vivo* has consequences that are in accordance with

decreased BDNF activity. Systemic nerve growth factor treatment, which leads to a condition resembling peripheral inflammation, raises BDNF levels in sensory neurons and increases nociceptive spinal reflex excitability. This increased central excitability is reduced by TrkB-Fc (Gustafsson et al., 2003). Moreover, intraventricular delivery of TrkB-Fc suppresses epileptogenesis, similar to what has been observed in heterozygous BDNF knockout mice and in transgenic mice overexpressing truncated TrkB receptors and with decreased endogenous BDNF levels (Lähteinen et al., 2002). In contrast to these data, TrkB/Fc chimera inhibits BDNF-induced cell proliferation of the BaF-TrkB-BD mouse pro-B cell line transfected with TrkB. The ED_{50} for this effect is typically 0.1-0.4 μg/mL in the presence of 16 ng/mL of recombinant human BDNF (Croll et al., 1998).



III. HYPOTHESIS AND OBJECTIVES

1. HYPHOTHESIS

Several signaling pathways coordinate the pre- and postsynaptic sites in the neuromuscular junction. This include that various presynaptic receptors seem to play an important role in the axonal competition leading to synapse loss. The final functional outcome of a synaptic contact can be built by the confluence of these receptor-mediated signaling on intracellular protein.

In previous results obtained by Histology and Neurobiology Unit at URV indicated that there is a release inhibition mechanism based on a mAChR-PKC-VDCC intracellular cascade. When it is fully active in certain weak motor axons, it can depress ACh release and even disconnect synapses. This mechanism plays a central role in the elimination of redundant neonatal synapses because functional axonal withdrawal can indeed be reversed by mAChR, PKC or VDCC block. This seems to indicate the involvement of these molecules in the process of synapse elimination. According with the previous results, it has been formulated the following hypothesis:

1.1. GENERAL HYPHOTHESIS

During the development an activity-dependent molecular mechanism of stabilization/unstabilization of the neuromuscular connections can operate by means of the convergence of multiple signaling pathways between the synaptic cells. This molecular mechanism includes the coordinated involvement of signaling through TrkB, mAChRs and ARs.

1.2. SPECIFIC HYPHOTHESIS

1. Individual presynaptic mAChR subtypes (M_1, M_2 and M_4), AR (A_1 and A_{2A}) and TrkB receptors are involved in the control of synapse elimination in the mouse NMJ.

2. The postnatal axonal elimination is regulated by the cooperation of several muscarinic and adenosine receptor subtypes.

3. The postnatal axonal elimination is regulated by the cooperation of several muscarinic receptor subtypes and TrkB receptor.

2. OBJECTIVES

2.1. GENERAL OBJECTIVE

The main objective of this thesis was to investigate the involvement of mAChRs, ARs and TrkB signaling in developmental synapse elimination and propose the coordinated molecular mechanism of participation and action at neuromuscular junction of mouse *Levator auris longus* muscle.

2.2. SPECIFIC OBJECTIVES

1. To know the effect of the individual mAChRs (M_1, M_2 and M_4), ARs (A_1 and A_{2A}) and TrkB signaling in the control of the synapse elimination during development. Identify how the modulation of these pathways modifies the number of motor nerve terminals per synapse at P7 and P9.

2. To know the effect of the individual mAChRs (M_1 and M_2), ARs and TrkB receptor signaling on the evolution of the number of motor nerve terminals per synapse at P15.

3. To determinate the involvement of mAChRs (M_1, M_2 and M_4), ARs (A_1 and A_{2A}) and TrkB receptor on maturation rate of nAChRs postsynaptic cluster at P7, P9 and P15.

4. To know the interaction between mAChRs (M_1, M_2 and M_4) and ARs (A_1 and A_{2A}) signaling in the modulation of the synapse elimination during development. To identify the number of motor nerve terminals per synapse at P7 and P9.

5. To know the interaction between mAChRs (M_1, M_2 and M_4) and TrkB signaling in the modulation of the synapse elimination during development. To identify the number of motor nerve terminals per synapse at P9.

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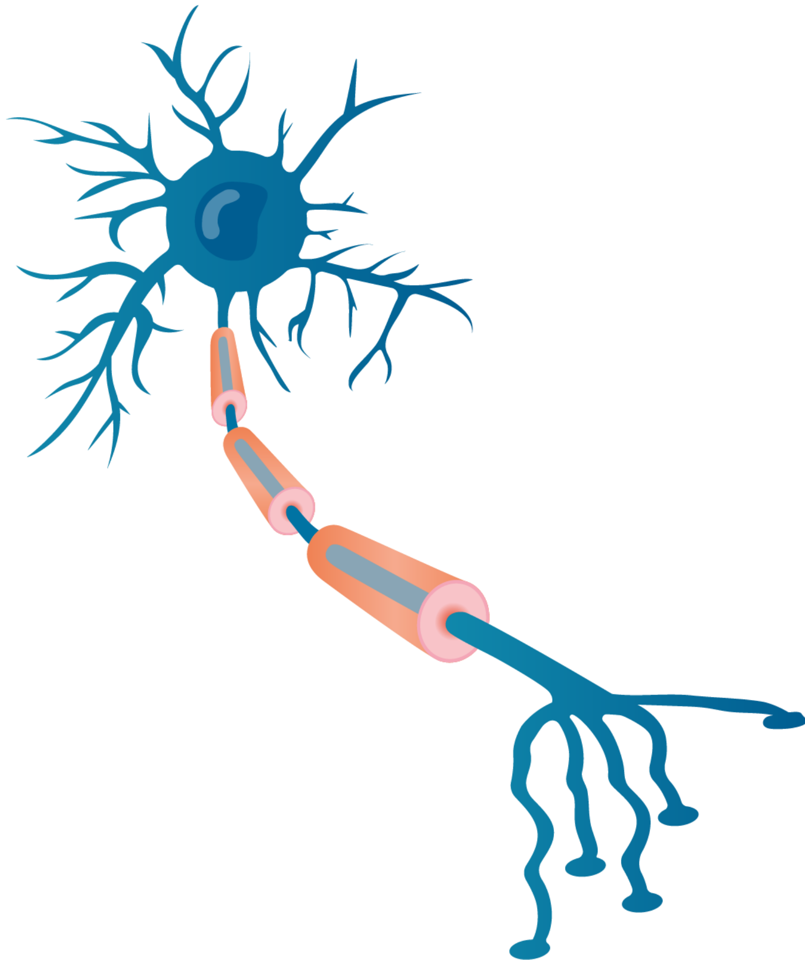
Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process
Laura Nadal Magriñà



III. MATERIAL AND METHODS

1. ANIMALS

To perform this thesis, a transgenic B6.Cg-Tg(Thy1-YFP)16Jrs/J mouse has been used (https://www.jax.org/strain/003709, The Jackson Laboratory; retrieved Sep 2016). This specific transgenic mouse contains a gene reporter and a regulatory sequence that directs its expression in neurons (**figure 10**).

The gene reporter used in this mouse is the yellow fluorescent protein (YFP), developed on the basis of GFP-like protein from jellyfish *Aequorea macrodactyla* (Xia et al., 2002). YFP possesses single excitation maximum at 508 nm, and emission maximum at 524 nm. Its use is mainly intended for protein labelling, which allows cell localization studies.

To direct the label of the YFP in neurons, the transgene construct contains the 5′ portion of the *Thy1* gene, extending from the promoter to the intron following exon 4. Thy1 is an immunoglobulin superfamily member that is expressed by projection neurons in many parts of the nervous system, as well as by several nonneuronal cell types, including thymocytes (Morris, 1985; Gordon et al., 1987).

Figure 10. Transgene construct of B6.Cg-Tg(Thy1-YFP)16Jrs/J mouse. In blue, the regulatory element derived of *Thy1* gene which direct the expression in neurons. In yellow, the yellow fluorescent protein (YFP) as a gene reporter provided the labelled in neurons.

The neural specificity of this promoter can be used to express YFP and label exclusively neurons. Vidal et al., 1990 demonstrated that neural and nonneural expression depend on distinct genomic elements. In concrete, a deletion of exon 3 and its flanking introns limits the expression in neural cells. Indeed, a construct lacking this intron has been successfully used to overexpress β-galactosidase and growth-promoting molecules in neurons with minimal nonneural expression (Kelley et al., 1994; Caroni, 1997)

B6.Cg-Tg(Thy1-YFP)16Jrs/J mouse has a wide range of neuron types labelled. Since motoneurons are the only neuron type in neuromuscular junction, this mouse becomes an excellent model for studying the neuromuscular synapse.

There are three features of YFP transgene expression that may be generally useful. First, multiple spectral variants express well in transgenic mouse. Second, long-term expression and repeated excitation of YFP are minimally toxic. Third, lines generated from identical or nearly identical transgenes exhibit distinct patterns of expression. Together, these features make it possible to label multiple neuronal subsets *in vivo* and to image them over protracted periods. These abilities, in turn facilitate studies of neuronal structure, function, and development in mammals (Feng et al., 2000).

B6.Cg-Tg(Thy1-YFP)16Jrs/J mouse has the advantages of YFP mouse described above. In concrete, this model has been widely used to study neuronal development and regeneration (Feng et al., 2000; Keller-Peck et al., 2001; Nguyen et al., 2002; Porrero et al., 2010). Thy1 levels in neurons increase markedly during early postnatal life, and thy1-derived transgenes have been reported to exhibit corresponding developmental regulation (Morris, 1985; Kollias et al., 1987; Caroni, 1997). As expected, levels of YFP increased postnatally in Thy1-YFP mouse. Nonetheless, in some lines, YFP expression was readily detectable in motor axons by embryonic day (E13), the stage at which neuromuscular junctions are just beginning to form (Noakes et al., 1993). This has made it possible to view the initial stages of synaptogenesis.

All experiments were conducted on Thy1-YFP-expressing mice. In some cases, the results have been check with wild-type C57BL/6J mice (https://www.jax.org/strain/000664, The Jackson Laboratory; retrieved Sep 2016). C57BL/6J is the most widely used inbred strain and the first to have its genome sequenced. Although this strain is refractory to many tumours, it is a permissive background for maximal expression of most mutations.

All mice were maintained on the animal facility in a standard cage of Makrolon^R (23x46x14 cm^3) and under standards conditions: constant temperature (22 ± 2ºC), relative humidity (50 ± 10%) and a 12-hour light/dark schedule. The feeding and the hydration of the animals has been *ad libitum*, with maintenance feed for mice VRF-1 by Charles River and chloride water. The use and treatment of animals has been made in accordance with *Llei 5/1995 and Decret 214/1997 de la Generalitat de Catalunya*, under the approval of *Comitè Ètic d'Experimentació Animal de la Facultat de Medicina i Ciències de la Salut de la Universitat Rovira i Virgil*.

Neonatal pups of either sex (4-30 days) were obtained and the date of birth was designated postnatal day 0 (P0). To minimize the variability in the measurements, the timing of conception has been monitored. Also, the weights of the individuals were within 5% of the mean for a given day after conception. The mice were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. After postnatal day 5 (P5), agonists and antagonists of the interested receptors were administrated (see in section 3 of material and methods). When the treatment finished in postnatal day (P7), postnatal day (P9) or postnatal day (P15), the mice were sacrificed. The first step of this procedure was the induction of anesthesia with 2% tribromoethanol (TBE; 0.15 ml/10 g body weight, I.P.). Once mice were deeply anesthetized (lacking the reflexes), proceeded to the handling and their sacrifice by making a cut in the jugular vein. Dissection procedures to obtain the tissue of interest (see below) were performed as soon as possible to avoid possible degradation processes.

Figure 11. B6.Cg-Tg(Thy1-YFP)16Jrs/J mice at different ages. A. Mouse postnatal day 9 (P9). **B**. Mouse postnatal day 15 (P15). **C**. Mouse postnatal day 30 (P30).

2. STUDY MODEL: NEUROMUSCULAR JUNCTION

Skeletal neuromuscular junctions have been used for many years to study neurotransmission, synaptic plasticity and competitiveness. It is an ideal model for some reasons: they are highly accessible, relatively simple, functionally uniform, and so much larger than central synapses that their size and shape can be assessed light microscopically (Sanes and Lichtman, 1999). Moreover, several studies have noted differences in neuromuscular structure between young adult and aged rodents and humans (Oda, 1984).

Synapse development and the regeneration can be modulated *in vivo* easily (Garcia et al., 2011; Andlauer and Sigrist, 2012). In addition, synapses between motoneurons and muscle cells can be studied and visualized by immunolabeling techniques using antibodies (Garcia et al., 2010f). Finally, gene expression could be altered and studied in detail by transgenic mice (Burden, 1977).

The NMJ is very useful in functional studies; unlike the central nervous system, it has a single neurotransmitter system, the cholinergic. This feature has made it a model widely used in electrophysiological studies (Keshishian et al., 1996; Santafé et al., 2003, 2009a; Zhang and Stewart, 2010).

In this thesis, the *Levator auris longus* muscle has been used as a model to study the neuromuscular junction. This muscle was described by Denise Angaut-Petit et al. (1987). It is located under the dorsal skin in the area of the head and neck and it is used to wiggle the ear. It has a cranial and caudal part. The fibers in the cranial region originate in the spines of the fourth cervical vertebrae and go towards anterior part of the pinna base, where they insert. The fibers in the caudal region extend from the fourth and fifth cervical vertebrae to the back of the pinna base. It consists of fast-twitch muscle fibers (Erzen et al., 2000) which are arranged in five or six layers of cells in the cranial portion (5.25 ± 0.78, Lanuza et al., 2003), while in the caudal part could be more (Angaut-Petit et al., 1987).

LAL muscle is subcutaneous, this peculiarity allows injected drugs *in vivo* to directly act on the surface muscle and, therefore, in the nerve endings. Due to the technical features, thin and few layers, makes it possible the effect and spread of the agents directly on muscle cells so inside the NMJ. It has a flat shape that allows a good visibility of the nerve endings of the auricular branches posterior of the facial nerve, which innervate the muscle that allows observing the motor plates without having to make sections (Tomàs et al., 2000; Lanuza et al., 2001).

The mouse LAL muscle is located in the back part of the neck and consists in two parts (**figure 12**): the left muscle (LAL 1) and the right muscle (LAL 2) separated by a midline. To minimize the number of animals sacrificed, both LAL muscles per mouse were used.

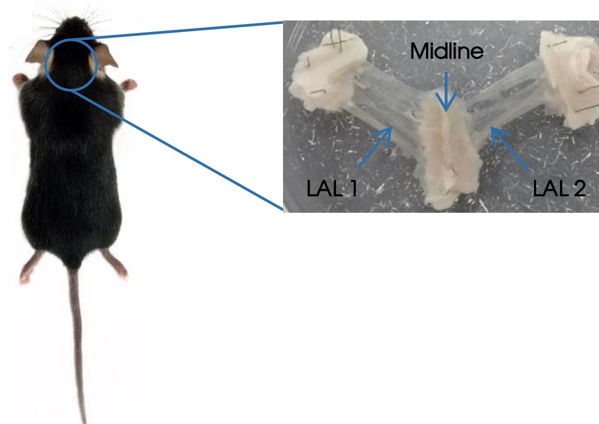


Figure 12. ***Levator auris longus*** **(LAL) muscle structure.** The LAL muscle is located under the dorsal skin in the area of the head and neck and consists in two parts: LAL 1 and LAL 2 separated by a midline.

The LAL muscle is an excellent model to study the NMJ morphology through immunohistochemistry experiments (see below). This is because it is a very thin muscle, and, therefore, the antibodies can penetrate and immunolabel the target proteins allowing the visualization of the nerve terminal and the nAChRs. Moreover, the LAL muscle is very superficial allowing that drugs in the process of drug treatment penetrated in the muscle surface easily without causing any stress to the animal.

3. RECEPTOR AGONISTS AND ANTAGONISTS

To study the effect of mAChRs, ARs and TrkB receptors in the synaptic elimination process, agonists and antagonists of these three types of receptors were used. Therefore, non-selective and selective agonists and antagonists were used to perform the inhibition or potentiation of the receptors. In the tables bellow, is shown the information about the name of the drug, the manufacturer, the function and finally the stock and work solution of each agonist and antagonist used.

3.1. MUSCARINIC RECEPTOR AGONISTS AND ANTAGONISTS

A non-selective muscarinic receptor agonist and antagonist were used. Moreover, three specific antagonists of muscarinic receptors M_1, M_2 and M_4 were used to perform more detail experiments.

Table 1. Information of mAChR agonists and antagonists.

Drug	Manufacturer	Function	Stock solution	Work solution
Oxotremorine M	Sigma O100	Non-selective agonist of muscarinic receptors	50 mM	1 μM
Atropine	Sigma A0132	Antagonist of muscarinic receptors	200 μM	2 μM
Pirenzepine	Tocris 1071	Selective antagonist of M_1 receptor	10 mM	10 μM
Methoctramine	Sigma M105	Selective antagonist of M_2 receptor	1 mM	1 μM
MT3	Alomone Labs M140	Selective antagonist of M_4 receptor	50 μM	100 nM

3.2. ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS

A non-selective adenosine receptor antagonist was used. The same way as muscarinic receptors, two selective antagonists of A_1 and A_{2A} receptors were used to specific inhibitions.

Table 2. Information of AR agonists and antagonists.

Drug	Manufacturer	Function	Stock solution	Work solution
Adenosine	Sigma A9251	Non-selective agonist of adenosine receptors	100 mM	25 μM
8-SPT	Sigma A013	Antagonist of adenosine receptors	100 mM	100 μM
DPCPX	Sigma C101	Selective antagonist of A_1	50 mM	100 nM
SCH-58261	Tocris 2270	Selective antagonist of A_{2A}	100 mM	50 nM

3.3. NEUROTROPHIN RECEPTOR ANTAGONIST

A selective neurotrophin receptor (TrkB) antagonist was used to block the TrkB-BDNF signaling pathway.

Table 3. Information of TrkB receptor antagonist.

Drug	Manufacturer	Function	Stock solution	Work solution
TrkB Fc chimera	R&D Systems 688-TK-100	Inhibition of BDNF and NT4	100 μg/ml	5 μg/ml

The stock solutions were made up with deionized water. Some stock solutions of these agonists and antagonists (DPCPX and SCH-58261) have to be diluted with dimethyl sulfoxide (DMSO). The final concentration of DMSO in control and drug-treated preparations was 0.1% (v/v). In control experiments, this concentration of DMSO did not affect any of the parameters studied (see in results). The working solutions were diluted with phosphate buffer saline (PBS).

4. METHODOLOGIES

4.1. INJECTION PROCEDURE

The different solutions containing the different agents affecting synaptic elimination process were administered by subcutaneous injection over the LAL muscle surface.

All solutions completely covered the LAL surface and remained in place a minimum of 2 hours before vascular reabsorption. Moreover, none inflammatory response could be detected. This was determined by recovering the LAL muscle with methylene blue.

The newborn mice were anesthetized with 2% tribromoethanol (0.15 ml/10 g body weight, I.P.) to obtain adequate immobilization and to reduce stress and pain. Under aseptic conditions, 50 µl of sterile physiological saline or DMSO and various solutions (antagonists and agonists of the considered receptors) were administered by subcutaneous injection over the LAL external surface as described above (Lanuza et al., 2001). To assist the injection of the drug it is useful the use of a forcep (**figure 13**). The solutions were administered at a concentration in accordance with the previously reported biological action of each substance (Santafé et al., 2004, 2015; Garcia et al., 2010d)

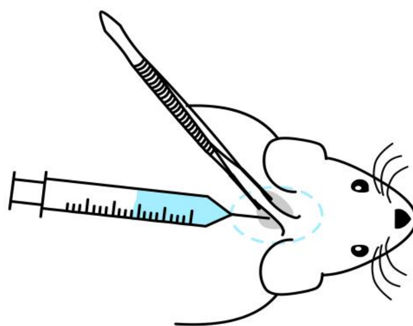


Figure 13. Injection procedure in *Levator auris longus* (LAL) muscle. With forcep helps, administrate 50 µl of phosphate-buffered saline (PBS) or drug agonist/antagonist by subcutaneous injection creating a small delimited bubble over the LAL muscle surface.

4.2. DRUG TREATMENT

Three types of postnatal stages were studied to evaluate the synaptic elimination process during the development of NMJ: P7, P9 and P15 (**figure 14**).

The animals received 2, 4 or 10 injections from postnatal day 5, and the LAL muscles were studied on days 7, 9 and 15. Pup mice were in a housing period between postnatal day 0 to day 5. After day 5 to days 7, 9 or 15 the animals were injected with agonists or antagonists of mAChRs, ARs and TrkB receptor. At day 7, 9 or 15 the mice were sacrificed for obtaining the LAL muscle in order to perform the morphological analysis.

In P7 experiments, mice received two injections on postnatal day 5 and 6 (one per day) and they were sacrificed at postnatal day 7. In P9 experiments, mice received four injections on postnatal day 5, 6, 7 and 8 (one per day) and they are sacrificed at postnatal day 9. Finally, in P15 experiments, mice received ten injections from postnatal day 5 to day 14 (one per day) and they are sacrificed at postnatal day 15.

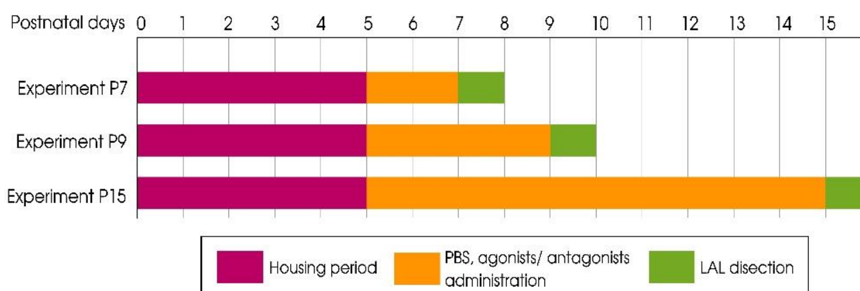


Figure 14. Timetable of drug treatment in mice. Three types of postnatal stages were studied: P7, P9 and P15. In purple, the housing period, in orange the drug administration and in green the day of the LAL dissection.

4.3. *LEVATOR AURIS LONGUS* MUSCLE DISSECTION

The mouse LAL is a thin and flat sheet of muscle located superficially on the dorsum of the neck. It is a fast-twitch muscle that functions to move the pinna. It contains rostral and caudal portions that originate from the midline of the cranium and extend laterally to the cartilaginous portion of each pinna. The muscle is supplied by a branch of the facial nerve that projects caudally as it exits the stylomastoid foramen. LAL muscle offers advantages for the investigation of *in vivo* effects of drugs on NMJs and muscles. First, its superficial location facilitates multiple

local applications of drugs. Second, its thinness (2-3 layers of muscle fibers) permits visualization and analysis of almost all the NMJs within the muscle. Third, the ease of dissecting permits the maintenance of the physiological conditions as long as possible. Last, and perhaps most importantly, a small applied volume easily covers the entire muscle surface, provides a uniform and prolonged exposure of all its NMJs to the drug and eliminates the need for a systemic approach.

For the muscle dissection, it is required a stereoscopic microscope and surgical equipment. Firstly, mice were anesthetized with an overdose of 2% tribromoethanol (0.15 ml/10 g body weight, I.P.). Death was assured by assessing lack of heartbeat. The second step was to pin down the mouse in a support, dorsal side up with one pin in each paw and one pin through the nose. The first incision was made through the skin only, using small spring scissors to cut from the beginning of the neck to reach the eyes obtaining T shape (step 1 **figure 15**). Carefully, the skin in this region was removed, but avoid cutting too close to the ears as this is one of the points of attachment for the LAL muscle. After, the head was excised in order to fix the whole sample (step 2 **figure 15**). The head containing the LAL was submerged in a solution with 4% paraformaldehyde during 90 min (step 3 **figure 15**). Using small spring scissors, 1cm to the right of the fat tissue (toward the left ear) was cut from the proximal edge of the left muscles until reaching the shoulder. The same procedure to the other side was performed. Once the incision was made, it has been peeled back the muscles connected with small forceps to expose the ventral side of the muscle. It has been trimmed the connective tissue and fascia while carefully pulling up on the muscles with forceps. It has been cut around the caudal end of the right muscles at the base of the ear. Continue cutting, moving towards the rostral end of the right ear, keeping the right muscle turned over. The same movements for the left part of the muscles were done. It was better to include more tissue at this step than risk damaging the LAL itself (step 4 **figure 15**). If the muscle began to dry out, pipette 1X PBS over the muscle. The dissected muscles was placed in a Petri dish with Sylgard containing 1X PBS, dorsal side up. Down four corners of muscle were pinned with small insect pins. Using small forceps and spring scissors, connective tissue from dorsal and ventral surfaces of the muscles was cleaned. It was important to locate the midline where the right and left LAL muscles converged. The muscle was fixed a second time with 4% paraformaldehyde during 45 min (step 5 **figure 15**). Afterwards, it has been done three washes of 5 minutes each and the muscle was turned over and re-pinned in order to clean off ventral

surface. The undesirable muscles were removed until it has been got deeper muscle which is the LAL (step 6 **figure 15**).

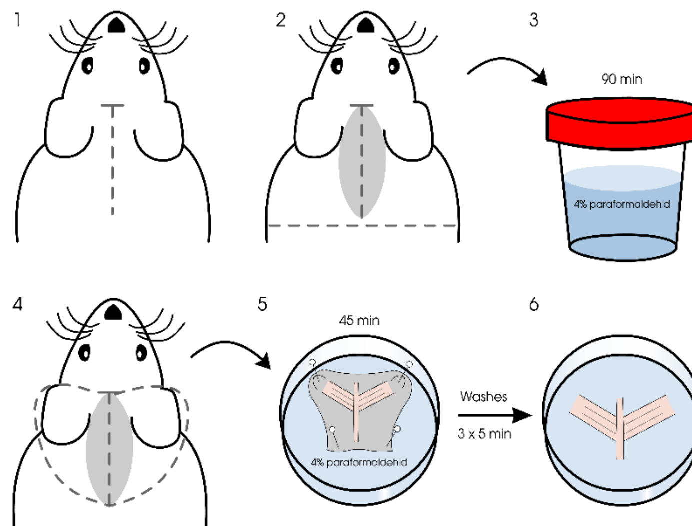


Figure 15. Dissection procedure in *Levator auris longus* (LAL) muscle. 1. Cut the skin obtaining a T shape. **2**. Cut the whole head. **3**. Fix whole head with 4% paraformaldehyde (90 min). **4**. Bind the tips of the T shape. **5**. Fix whole muscles with 4% paraformaldehyde (45 min). **6**. Make three PBS washes of 5 min each one and removed the undesirable muscles and extra tissue until LAL muscle is isolated.

4.4. IMMUNOHISTOCHEMISTRY

4.4.1. BASIS OF THE TECHNIQUE

Fluorescent immunohistochemistry (IHC) is a technique of monoclonal as well as polyclonal antibodies to determine the tissue distribution of an antigen of interest with high precision. The classical IHC assay involves detection of epitopes expressed by a single protein-target within a tissue sample using a 'primary antibody' capable of binding those epitopes with high specificity. After the epitope-antibody binding event, a 'secondary antibody' capable of binding the primary antibody with high specificity is added. The secondary antibody is coupled to a fluorophore, a molecule which has the ability to absorb light energy of a specific wavelength and re-emits light at a longer wavelength. There are a lot of number of fluorophores which have different properties of excitation and emission. For this reason, it was possible to label different proteins in different colors and display them together.

4.4.2. BUFFERS AND SOLUTIONS USED IN IMMUNOHISTOCHEMISTRY

- **Phosphate Buffered Saline** (PBS). Composed by NaCl 140 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM and Na_2HPO_4 8.1 mM with adjusted pH at 7.4.
- **Blocking and permeability buffer**. Composed by 4% of Bovine Serum Albumin (BSA) and 0.1% of Triton X-100.
- **Paraformaldehyde.** Working solution: 4% dissolved in saline solution.
- **Mowiol.** Mixed with glycerol and 0.2M Tris-HCL pH 8.5.
- **p-phenylenediamine**. 0.1% (0.001 g/ml Mowiol).

All the salts used to prepare the PBS solution were from Sigma. Also the BSA, Triton X-100, paraformaldehyde and p-phenylenediamine were from Sigma. Mowiol was from Calbiochem.

4.4.3. ANTIBODIES AND TOXINS IN INMMUNOHISTOCHEMISTRY

For immunolabeling the muscles, primary antibodies were used with their corresponding secondary antibodies and a widely used toxin to label acetylcholine receptors, α-bungarotoxin (α-BTX). The information of each antibody and toxin is described in the table below (**table 4-6**).

Table 4. Primary antibodies used in immunohistochemistry technique.

Antibody	Manufacturer	Source	Dilution
Neurofilament	N4142; Sigma	Rabbit	1:1000
Neurofilament	N0142; Sigma	Mouse	1:1000
Syntaxin	S0664; Sigma	Mouse	1:1000
S-100	Z0311; Dako Cytomation	Rabbit	1:1000

Table 5. Secondary antibodies used in immunohistochemistry technique.

Antibody	Manufacturer	Source	Dilution
Alexa-fluor 488	A21206; Molecular Probes	Donkey α-rabbit	1:300
Alexa-fluor 488	A21202; Molecular Probes	Donkey α-mouse	1:300
Alexa-fluor 647	A31573; Molecular Probes	Donkey α-rabbit	1:300

Table 6. Neurotoxin used in immunohistochemistry technique.

Fluorochrome	Manufacturer	Source	Dilution
TRICT	T1175; Molecular Probes	α-BTX	1:800

4.4.4. TISSUE PREPARATION

The tissue preparation in order to perform the quantification analysis was different depend on the mouse strain that it has been used. For C57BL/6J mice, after removing the LAL muscle and washing it in PBS, the muscles were incubated with a blocking and permeability buffer. This buffer was composed by 4% of BSA and 0.1% of Triton X-100. Afterwards, the muscle could be labelled with some antibodies or toxins to detect the proteins of interested. In this case, it was needed to perform the immunohistochemistry technique to label the nerves terminal and nAChRs. For transgenic B6.Cg-Tg(Thy1-YFP)16Jrs/J mice was only necessary to label the nAChRs (see bellow).

4.4.5. LABELING OF NMJ

In order to study the neuromuscular junction, it is possible to label each of its cells or components (nerve terminal, nAChRs and Schwann cell) by immunohistochemistry. For the morphological studies of this thesis, it was only needed to detect the pre- and the postsynaptic components. **Figure 16** shows a confocal image of the three components distribution. The nerve terminal was detected by neurofilament or syntaxin labeling protein in green. Syntaxin is a protein exclusively located in ACh vesicles membrane which is ready to be release when the action potential arrives at the nerve terminal. Whereas neurofilaments are intermediate filaments specific of MNs. The Schwann cell was labelled by S-100 in blue which is a protein which belongs to a superfamily of calcium-binding proteins that are involved in regulation of protein phosphorylation, transcription factors, Ca^{2+} homeostasis, the dynamics of cytoskeleton constituents, enzyme activities, cell growth and differentiation, and the inflammatory response (Donato et al., 2003). In **figure 16**, it has been used the primary antibodies anti-neurofilament or anti-syntaxin and anti-S100. The primary antibodies dilutions were shown in **table 4**.

The secondary antibodies are against the specie which were obtained the primary antibodies. In **figure 16**, Alexa fluor 488 donkey anti-mouse was used to detect neurofilament or syntaxin protein while Alexa fluor 647 donkey anti-rabbit to detect S-100. The secondary antibodies dilutions were shown in **table 5**.

The postsynaptic component was labelled in red by a toxin, α-bungarotoxin conjugated with tetramethyl rhodamine iso-thiocyanate (TRICT)

which was joined irreversibly to nAChRs and therefore allows us to identify the synaptic area. The dilution of neurotoxin was shown in **table 6**.

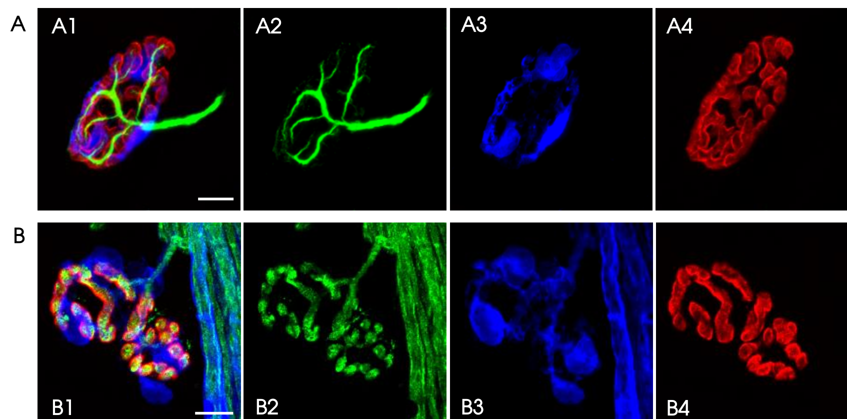


Figure 16. Examples of adult neuromuscular junctions (NMJ) labelled by conventional immunohistochemistry. A. Nerve terminal labelled with neurofilament. **A1**. The merge of the three labelled proteins: neurofilament, S-100 and nAChRs. **A2**. In green, the nerve terminal labelled with neuroflament. **A3**. In blue, the Schwann cell labelled with S-100 and **A4** in red, the nAChRs labelled with α-BTX-TRICT. **B**. Nerve terminal labelled with syntaxin. **B1**. The merge of the three labelled proteins: syntaxin, S-100 and nAChRs. **B2**. In green, the nerve terminal labelled with syntaxin. **B3**. In blue, the Schwann cell labelled with S-100 and **B4** in red, the nAChRs labelled with α-BTX-TRICT. Scale bar = 10 µm.

In this thesis, we used two strains (Thy1-YFP and C57BL/6J) in order to study the synaptic elimination process at the NMJ. The specific approaches used for each strain is detailed below. In Thy1-YFP LAL muscles only the label with α-BTX-TRICT is needed because they contain the YFP transgene expression whereas in C57BL/6J muscles two proteins have to be labelled: the nerve terminal and the nAChRs.

4.4.5.1. PROCEDURE IN B6.Cg-Tg(Thy1-YFP)16Jrs/J MICE

After LAL isolation, Thy1-YFP LAL muscles were incubated in PBS containing a 1/800 dilution of 1µg/ml TRICT conjugated α-BTX (Molecular Probes) for 2h at room temperature. After that, three washes of PBS were needed for remove the excess of α-BTX. Finally, the muscles were mounted in Mowiol (Calbiochem) with 0.1% of p-phenylenediamine (Sigma) to extend the labelling. **Figure 17** shows a schematic diagram of the experimental procedure in Thy1-YFP mice.

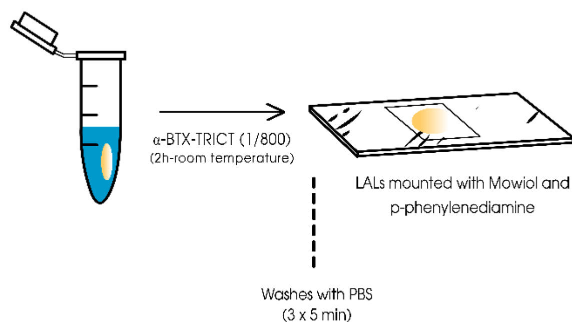


Figure 17. Procedure in Thy1-YFP mice. In Thy1-YFP mice whose nerve terminal was already labelled, it was only necessary to incubate LAL muscles with α-bungarotoxin-TRICT during 2 hours at room temperature, to label nAChRs. Three washes of PBS were needed to remove the excess of α-BTX-TRICT. The last step is mounted the LAL muscle with Mowiol and 0,1% of p-phenylenediamine.

When the procedure in Thy1-YFP muscles were done and LALs were mounted with Mowiol solution, the preparation was ready to be analyzed. It is important to wait until the preparation was dried in order to not lose the sample when it is placed in inverted fluorescence microscopy. The **figure 18** shows some confocal images from Thy1-YFP LAL muscle.

Protein	Primary antibody	Neurotoxin	Fluorochrome
nAChR	-	α-BTX	TRICT

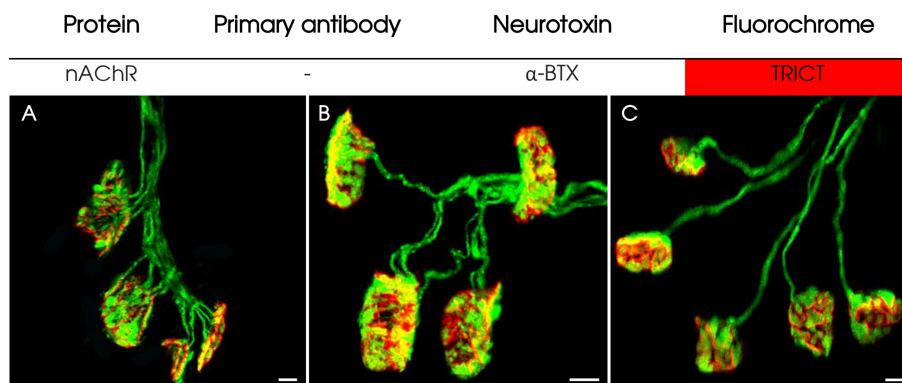


Figure 18. Confocal images from Thy1-YFP mice labelled with nicotinic acetylcholine receptors (nAChR). Nerve terminal and nAChR labelled. **A**. Example of multiinervated synapses at P7. **B**. Example of monoinnervated and multiinervated synapses at P9. **C**. Example of single innervated synapse at P15. Scale bar = 10 µm.

4.4.5.2. IMMUNOHISTOCHEMISTRY PROCEDURE IN C57BL/6J MICE

In this type of mouse, an immunofluorescence was performed on the LAL muscles. **Figure 19** shows a schematic diagram of the experimental procedure in C57BL/6J mice. As shows **figure 19**, after LAL fixation, the muscles were incubated overnight (O/N) at 4ºC with a permeation solution of 4% of BSA and 0.1% of Triton X-100 to block the unspecific bindings. Whole mounts of LAL were processed to detect the axons with an antibody against 200-kD neurofilament protein (1:1000; Sigma) and postsynaptic nAChRs with α-BTX-TRITC (1:800; Molecular Probes). Muscles were incubated O/N at 4ºC only with the rabbit antibody against 200-kD neurofilament (1:1000; Sigma) in 4% BSA and 0.1% Triton-X. The antibody against 200-kD neurofilament was widely used in our experiments because is a specific antibody for the immunocytochemical localization of neurofilaments with molecular weights of 200 kD in cultered cells or tissue preparations (Besalduch et al., 2010; Bowerman et al., 2010). Before adding the secondary antibody, it is important to make 3 washes of 5 minutes each one with PBS and shaking at room temperature. The appropriate secondary antibody (conjugated with Alexa-fluor 488) donkey anti-rabbit (1:300; Molecular Probes) was incubated for 4h at room temperature. The postsynaptic nAChRs were detected by α-BTX conjugated with TRICT. After the antibody incubation, the excess of the antibody was removed by washing the muscles with PBS. Whole muscles were mounted in Mowiol (Calbiochem) with 0.1% of p-phenylenediamine (Sigma).

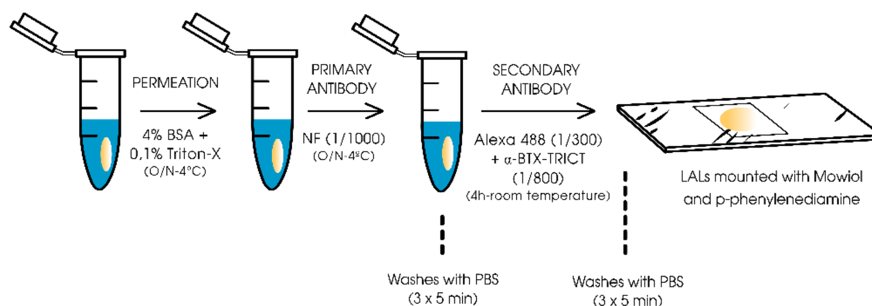
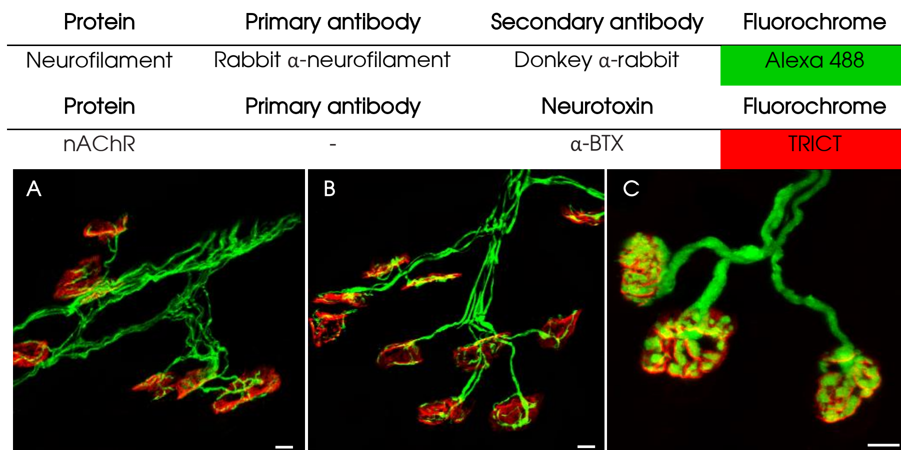


Figure 19. Immunohistochemistry procedure in C57BL/6J mice. The first step was to make a permeation with 4% of BSA and 0.1% of Triton X-100 (O/N at 4ºC). The follow day, it has been started the label of the nerve terminal with a dilution of 1/1000 of neurofilament (O/N at 4ºC). To eliminate the excess of the antibody, three washes with PBS (5 minutes per each wash) were done. The secondary antibody, Alexa 488 (1/300) and the α-bungarotoxin-TRICT is added (4h at room temperature). Three more washes with PBS were needed before mounted the LALs. As Thy1-YFP procedure, the last step is mounted the LALs muscle with Mowiol and 0.1% of p-phenylenediamine.

As Thy1-YFP LAL muscles, after the whole procedure, muscles could be studied by morphological analysis. The result is a NMJ labelled in green showing the neurofilament and in red showing the nAChRs. It is possible to merge the two colors to see the whole immunolabelled muscle. The **figure 20** shows some confocal images from C57BL/6J LAL muscle.



Protein	Primary antibody	Secondary antibody	Fluorochrome
Neurofilament	Rabbit α-neurofilament	Donkey α-rabbit	Alexa 488
Protein	**Primary antibody**	**Neurotoxin**	**Fluorochrome**
nAChR	-	α-BTX	TRICT

Figure 20. Confocal images by conventional immunohistochemistry (neurofilament and nAChR label) from C57BL/6J mice. A. NMJs at P7 where the majority of the synapses are multiinnervated. **B**. NMJs at P9 where some of the synapses remain multiinnervated and some of them are monoinnervated. **C**. NMJs at P15, most of the axons are single innervated. Scale bar = 10 µm.

The secondary antibody specificity was tested by an incubation in the absence of primary antibody. At least three muscles were used as negative controls (see in **figure 21**).

Figure 21. Negative control by immunohistochemistry in neonatal muscles. In the absence of primary antibody, Alexa 488 donkey anti-rabbit does not show any unspecific label. Scale bar = 10 µm.

4.5. VISUALIZATION BY FLUORESCENCE MICROSCOPY

Once the immunohistochemistry was done, NMJs were analyzed using an inverted Nikon TE-2000 fluorescence microscope (Nikon, Tokyo, Japan) connected to a personal computer running image analysis software (ACT-1, Nikon).

This fluorescence microscope allowed us to see the different proteins of NMJ which were previously labelled by immunohistochemistry or by YFP. When the NMJs were studied by fluorescent microscopy each protein could be identified in a specific color: nerve terminal in green (**figure 22B**) and nAChRs in red (**figure 22C**). To see the colocalization of two proteins both images were merged (**figure 22A**).

The images obtained were very clear, allowing the counting of the number of axons that innervated a muscle fiber as well as the changes on the maturation stage of these nAChRs. For illustration, **figure 22** shows two polyinnervated synapses in developing process. **Figure 22B** shows the feasibility of counting axons and **figure 22C**, the feasibility of determining the maturation stage of the postsynaptic plaque.

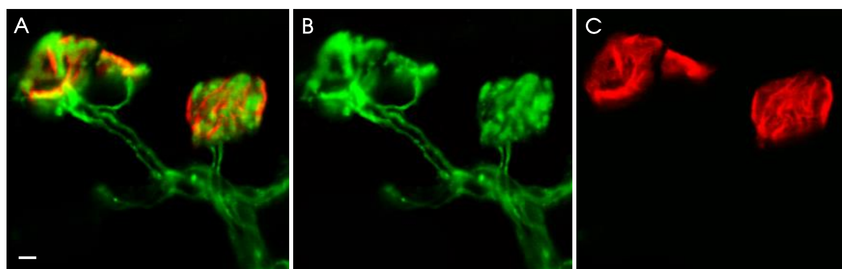


Figure 22. Fluorescence microscopy images of neuromuscular junctions (NMJ) from Thy1-YFP mice. A. The merge of the two NMJ components: the nerve terminal labelled with YFP and the nAChRs labelled with α-BTX-TRICT. **B.** YFP label shows several synapses: two of them with two axons and the other one with one axon. **C**. nAChRs labelled with α-BTX-TRICT show two types of maturation stages: on the left the nAChRs are in maturation stage 3 (MS3) and on the right the nAChRs are in maturation stage 2 (MS2).

4.6. CONFOCAL MICROSCOPY

For capturing images of NMJs, were used an inverted Nikon TE-2000 confocal fluorescence microscope (Nikon, Tokyo, Japan) connected to a personal computer running image analysis software (EZ-C1, Nikon).

High-resolution confocal images were obtained with a 63x oil objective (1.4 numerical aperture). Z stacks were obtained at 0.5-μm step size for depths of 20-40 μm, and additional optical sections above and below each junction were collected to ensure that the entire synapse was included.

By scanning many thin sections through the sample, confocal three-dimensional reconstructions of labelled axons in these mice permitted visualization of the complete arbor of individual motor axons. Using this method, representative images were captured in order to demonstrate the morphology of the neuromuscular synapses in Thy1-YFP and C57BL/6J mice. **Figure 23** shows confocal images from P9 C57BL/6J.

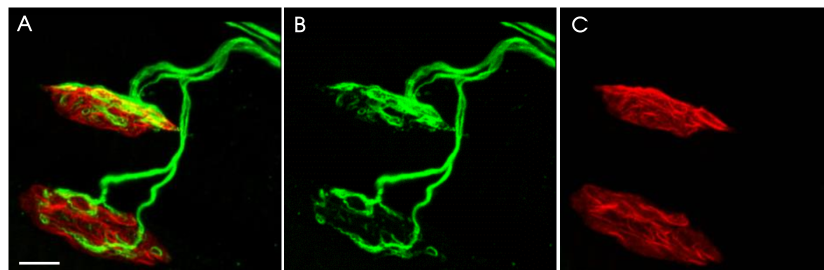


Figure 23. Confocal microscopy images from P9 C57BL/6J mice. A. The merge of the two labelled proteins: neurofilament and nAChRs. **B**. Neurofilament labelled. **C**. nAChRs labelled with α-BTX-TRICT. Scale bar = 10 μm.

4.7. MORPHOLOGICAL ANALYSIS

In both strains (Thy1-YFP and C57BL/6J), each muscle was analyzed by recounting the number of axons which innervate an endplate and by the maturation morphology of the nAChRs. The recounting was made randomly and blindly. For each muscle was counted a minimum of 100 synapses.

4.7.1. RECOUNT OF AXON NUMBER

The number of axons per each endplate was counted. Because of the difficulty in determining the exact number of axonal inputs for each nAChR cluster when more than two axons converge at the same synaptic site, the NMJs were classified into three groups: monoinnervated junctions, doubly innervated junctions, and triply or more innervated junctions (**figure 24**). This classification enabled us to calculate two parameters.

- **Axonal input percentage**: dividing the number of synapses of each group (1 axon, 2 axons and ≥3 axons) by the total number of synapses in the preparation and multiplying that result by one hundred.
- **Average number of axonal inputs**: the display of the three percentages of axonal input.

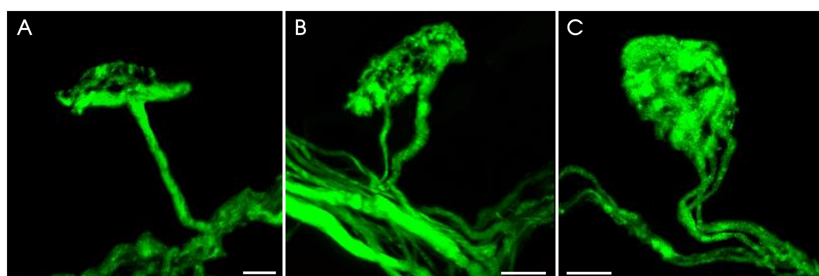


Figure 24. Classification of the number of axons in the study of synaptic elimination process. A. Represents a motor endplate innervated for one axon. **B**. Two axons innervated the same cluster of nAChRs. **C**. Three or more axons, in this case, three axons innervated the endplate. Scale bar = 10 μm.

4.7.2. MORPHOLOGIC MATURATION OF nAChRs

During development there is a redistribution of nAChRs that allow us to classify the nAChRs in different maturation stages. To determine the effect of different treatments on the maturity of nAChR clusters at the NMJ during the period in which polyneuronal innervation is being eliminated, the maturation of the clusters was divided into four morphological maturation stages (MS1–MS4) on the basis of criteria from previous studies of developing mammalian NMJs (Steinbach, 1981; Slater, 1982b; Lanuza et al., 2002) (**figure 25**).

- **MS1**: Uniform nAChR oval plaque with an indistinct boundary seen in the majority of NMJs at birth. A uniformly distributed porosity can be observed within this plaque.
- **MS2**: nAChR elongated oval plaque with a few hints of inhomogeneities in receptor density. The nAChRs are denser on a few narrow ridges within the plaque.
- **MS3**: An oval nAChR plaque with one or more fluorescence-free 'holes'. These holes are not innervated.
- **MS4**: The oval nAChR areas have been transformed into a more mature branched pattern with a moderately convoluted external border and high and low receptor density areas. The edge of the holes usually has a high density of receptors.

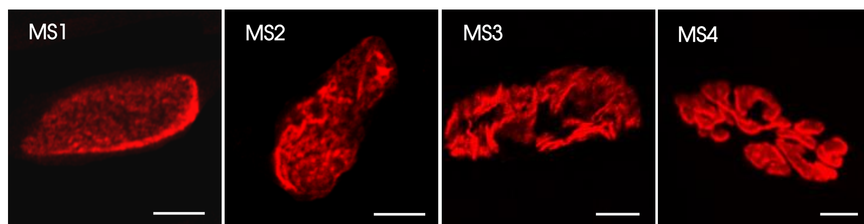


Figure 25. Classification of morphological maturation stages (MS1-MS4) of nicotinic acetylcholine receptors (nAChR) in the study of synaptic elimination process. MS1. Represents a uniform nAChR plaque. **MS2**. Is characterized for having a few hints of inhomogeneities in receptor density. **MS3**. The emergence of holes is present. **MS4**. The edge of the holes usually has a high density of receptors. Scale bar = 10 µm.

All this information was collected in a template (**figure 26**): basic information of the preparations which it has been analyzed, number of axons and the stage of maturation of nAChR. Finally, the total number of synapses and the percentage of multinnvertation (mentioned above) were noted.

Preparation code:
Analysis day:
Newborn age:
LAL number:

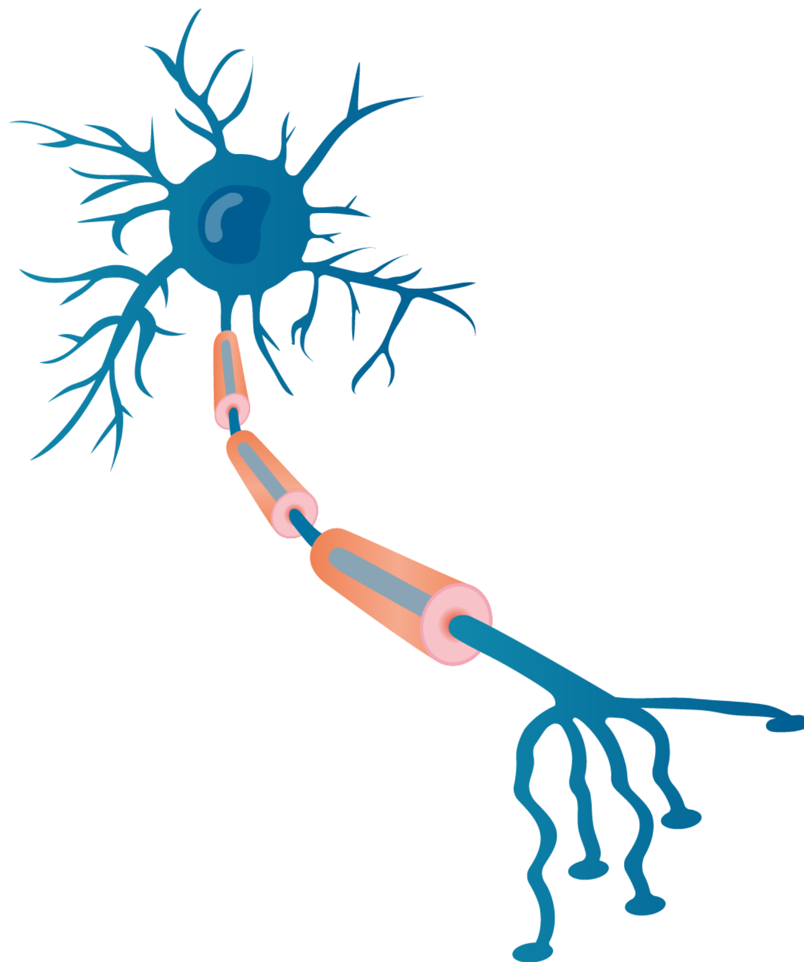
		MS1	MS2	MS3	MS4
1 axon					
2 axons					
3 or more axons					

Total of synapses: .... % multinnervation..............

Figure 26. Recount template. Template used to count presynaptic component (1 axon, 2 axons or 3 or more axons) and postsynaptic component (MS1, MS2, MS3 or MS4). On the top of the page fill basic details of the preparation (code, day, age…) and finally, fill the total number of synapses and the % of multinnervation.

4.8. STATISTICS ANALYSIS

All NMJs visible in their entirety were scored. A minimum of 100 NMJs per muscle were analyzed. At least six muscles were studied for each age and condition examined. Fisher's test was applied to compare percentages. The criterion for statistical significance was $P < 0.05$. The significance has been represented as following: *, §, † for $P < 0.05$; **, §§, †† for $P < 0.01$ and ***, §§§, ††† for $P < 0.005$. The categories were scored and the counting was performed with no knowledge of the age or treatment of the animals. The data are presented as mean ± SD.



IV. RESULTS

The results of this thesis have been structured in three chapters. The first chapter shows the involvement of individual mAChR subtypes (M_1, M_2 and M_4), AR subtypes (A_1 and A_{2A}) and TrkB receptor in the control of synapse elimination using agonists and antagonists of these receptors. The second chapter refers to the study of additive or occlusive effects of the inhibitors of two of these receptor sets, mAChR and AR and thus the existence of real cooperation between them in synapse elimination at the NMJ. Finally, the third chapter concerns whether the mAChR subtypes and the TrkB receptor work together and whether the respective pathway inhibitors have any additive or occlusive effects that reflect if there is any real cooperation between them in synapse elimination at the NMJ.

The following table shows the relation between the chapters and the content.

CHAPTER	CONTENT
CHAPTER 1	Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes), adenosine receptors (A_1 and A_{2A}) and tropomyosin-related kinase B receptor (TrkB) modulate in the developmental synapse elimination process at the neuromuscular junction.
CHAPTER 2	Synergistic action of presynaptic muscarinic acetylcholine receptors and adenosine receptors in developmental axonal competition at the neuromuscular junction.
CHAPTER 3	Presynaptic muscarinic acetylcholine receptors and TrkB receptor cooperate in the elimination of redundant motor nerve terminals during development.

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Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process
Laura Nadal Magriñà

CHAPTER 1. Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes), adenosine receptors (A_1 and A_{2A}) and tropomyosin-related kinase B receptor (TrkB) modulate in the developmental synapse elimination process at the neuromuscular junction.

This chapter corresponds to the published article (see Appendix I):

Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Marta Tomàs, Maria A. Lanuza, Manel M. Santafe, Josep Tomàs. (2016). Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes), adenosine receptors (A_1 and A_{2A}) and tropomyosin-related kinase B receptor (TrkB) modulate in the developmental synapse elimination process at the neuromuscular junction. Molecular Brain 9:67. DOI: 10.1186/s13041-016-0248-9.

1.1. BRIEF INTRODUCTION AND SCOPE

As described in the introduction, in newborn animals, the skeletal muscle fibers are polyinnervated by several motor axons (Ribchester and Barry, 1994) but at the end of the axonal competition, the endplates are innervated by a single axon. The NMJ has long been used as a model for studying the general principles of synapse development in an attempt to understand the synapse elimination process (Liu et al., 1994; Nguyen and Lichtman, 1996; Chang and Balice-Gordon, 1997; Sanes and Lichtman, 1999; Herrera and Zeng, 2003; Nelson et al., 2003; Wyatt and Balice-Gordon, 2003; Buffelli et al., 2004).

Various presynaptic receptors seem to play an important role in the axonal competition leading to synapse loss in the NMJ. The involvement of mAChRs in the elimination process may allow direct competitive interaction between nerve endings through a differential activity-dependent ACh release. It has been suggested that this mechanism plays a central role in the elimination of redundant neonatal synapses because functional axonal withdrawal can indeed be reversed by mAChR, PKC or VDCC block (Santafé et al., 2009a; Tomàs et al., 2011). However, local differential effectiveness and differential activity will determine eventual success, since an axon that fails at one synapse may be successful at another (Keller-Peck et al., 2001), which suggests complex regulation involving other receptors and postsynaptic- (and glial cell) derived factors. Both NTR and AR belong to leading presynaptic signaling pathways. In the adult NMJ, the activity of

one of these receptors can modulate a given combination of spontaneous, evoked and activity-dependent release conditions and a close dependence between them exist (Tomàs et al., 2014). These receptors and their intracellular signaling may help to refine the molecular and structural organization of the newborn synapses so that they can acquire their mature form. In this chapter, it has been shown the involvement of individual mAChR subtypes (M_1, M_2 and M_4), AR subtypes (A_1 and A_{2A}) and TrkB receptor in the control of synapse elimination using agonists and antagonists of these receptors.

1.2. RESULTS

1.2.1. POSTNATAL ELIMINATION OF NERVE TERMINALS

1.2.1.1. Normal evolution of postnatal polyneuronal innervation in the NMJ

Over the first several weeks of postnatal life, rodent motor axons remove branches. Each neuromuscular junction undergoes a transition from innervation by multiple converging axons to innervation by only one axon. The number of muscle fibers innervated by an axon decreases substantially and all but one input is eliminated from each fiber.

The **figure 27** shows the average axonal connections in the first 30 postnatal days (P). The average were counted in LAL muscle preparations from B6.Cg-Tg(Thy1-YFP) –from now YFP- and C57BL/6J mice. It has been represented also in this figure previous data (Lanuza et al., 2001, 2002; Santafé et al., 2001; Nelson et al., 2003) from *Sprague-Dawley* (SD) rats to emphasize similarities in rodents.

The results show that at P0, the average number of axonal connections in the three different models is 3, whereas at P7 is around 2 in mice and slightly less in rats. At P9, the average of the three animal models is almost the same (around 1,5-1,75) and matches at P30 when the average reach 1.

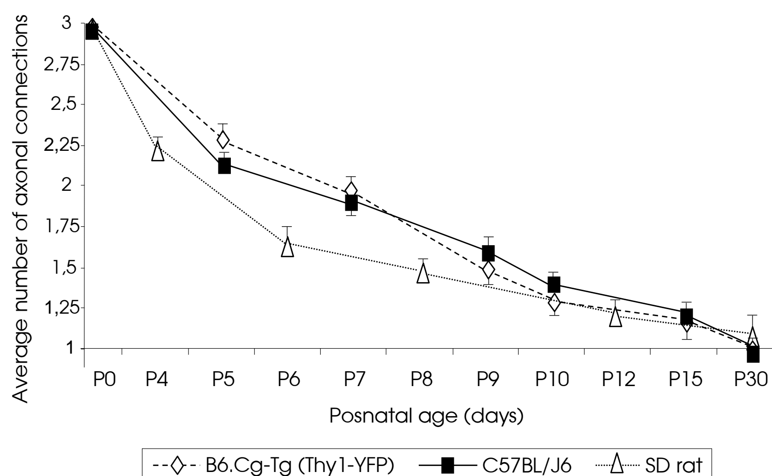


Figure 27. Postnatal evolution of polyneuronal innervation. Comparison of the results of axon counts in confocal immunohistochemistry LAL preparations of YFP and C57BL/6J mice and *Sprague-Dawley* (SD) rats.

In order to know the effect of the subcutaneous injection procedure on neuromuscular junction innervation, the results between YFP mice injected with PBS and YFP control mice (not injected) were compared. The histogram in **figure 28** shows the percentage of singly-, dually- and triply (or more)-innervated synapses in YFP animals at the considered postnatal days without any experimental manipulation (control), and also at P7, P9 and P15 after two (days 5, 6), four (days 5-8) and ten (days 5-14) subcutaneous PBS applications respectively (control PBS).

The values were shown that neither any significant difference between PBS and non-PBS preparations is observed ($p>0.05$) nor the drugs diluted with DMSO did not affect any of the parameters studied. About half of NMJs become monoinnervated at the end of the first postnatal week and almost all of them at P30.

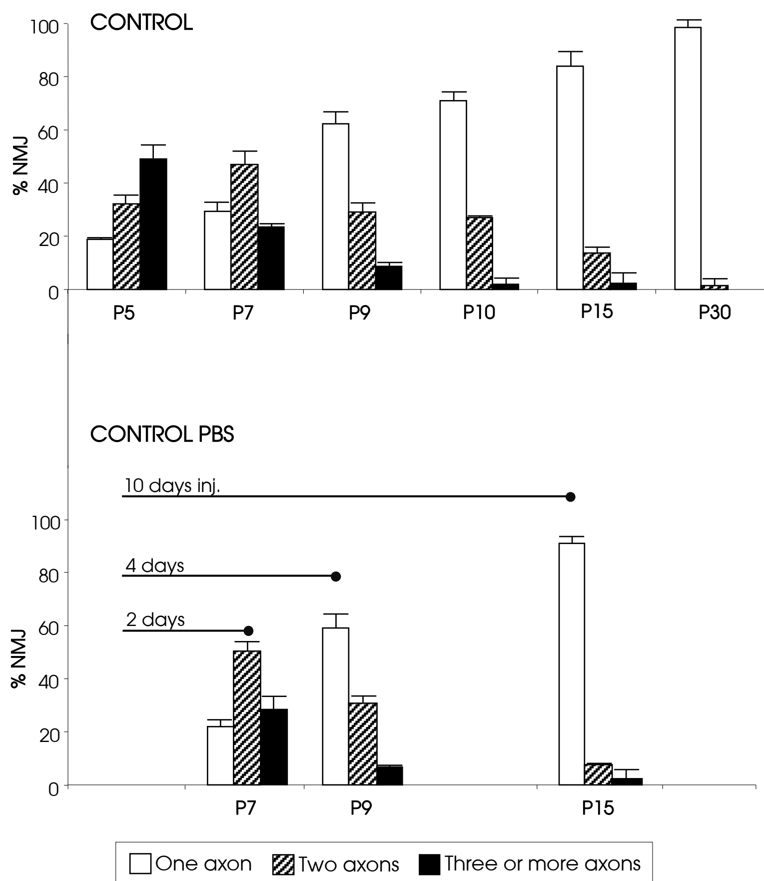


Figure 28. Injection effect in % neuromuscular junction (NMJ). **Control**: percentage of singly-, dually- and triply- (or more) innervated synapses in YFP animals on the postnatal days studied (P5, P7, P9, P10, P15 and P30) without any experimental manipulation (no injected). **Control PBS**: percentage of singly-, dually- and triply- (or more) innervated synapses in YFP animals at P7, P9 and P15 after two, four and ten daily subcutaneous PBS applications respectively. No differences are observed between PBS and non-PBS preparations ($P>0.05$).

Moreover, all the terminal branches of individual labelled motor axons were studied by montaging confocal image stacks. **Figure 29** shows a fragment of the terminal innervation by confocal immunofluorescence on the LAL muscle with mono - and polyinnervated NMJ from YFP and C57BL/6J mice at P9. In all cases, motor axons projected to a circumscribed subregion of the endplate band.

YFP mice express axons in green and nAChRs were labelled with α-BTX-TRICT whereas in C57BL/6J mice were labelled with neurofilament in order to label the nerve terminal and with α-BTX-TRICT to detect the nAChRs. Both mice allowed us to study nerve terminals and nAChRs to perform the recounting procedure.

At P9, the process of synaptic elimination is ongoing, thus the progressive transition from multiple to single innervation was shown. Thus, there was gradual loss of multiple innervation, so that some neuromuscular junctions remain multiply innervated for nearly two postnatal weeks, while other junctions lose their multiple innervation in several days. This variation in the time to completion was probably because the loss of multiple innervation was also gradual in another sense: each individual junction gradually loses multiple innervation.

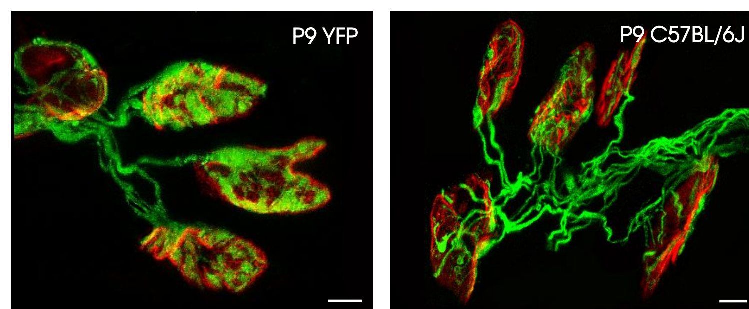


Figure 29. Confocal reconstruction of neuromuscular junctions in a *Levator auris longus* muscle from YFP and C57BL/6J mice at P9. Note that the YFP containing axons (green) project to all junctions and completely occupy the high-density nAChR clusters (red, labeled with rhodamine α-bungarotoxin), indicating that all the motor axons express YFP. The same label could be seen in C57BL/6J immunohistochemistry. Scale bar: 10 µm.

The **figure 30** shows some representative confocal immunofluorescence images of singly- and polyinnervated NMJ from YFP (autofluorescent axons) and C57BL/6J mice (axons stained with anti-neurofilament fluorescent antibody). The pictures show the feasibility of counting with precision the axon number in both preparations. Neuromuscular junctions innervated by one axon at different stages of development are shown in **figure 30A**, by two axons in **figure 30B** and by three or more axons in **figure 30C**.

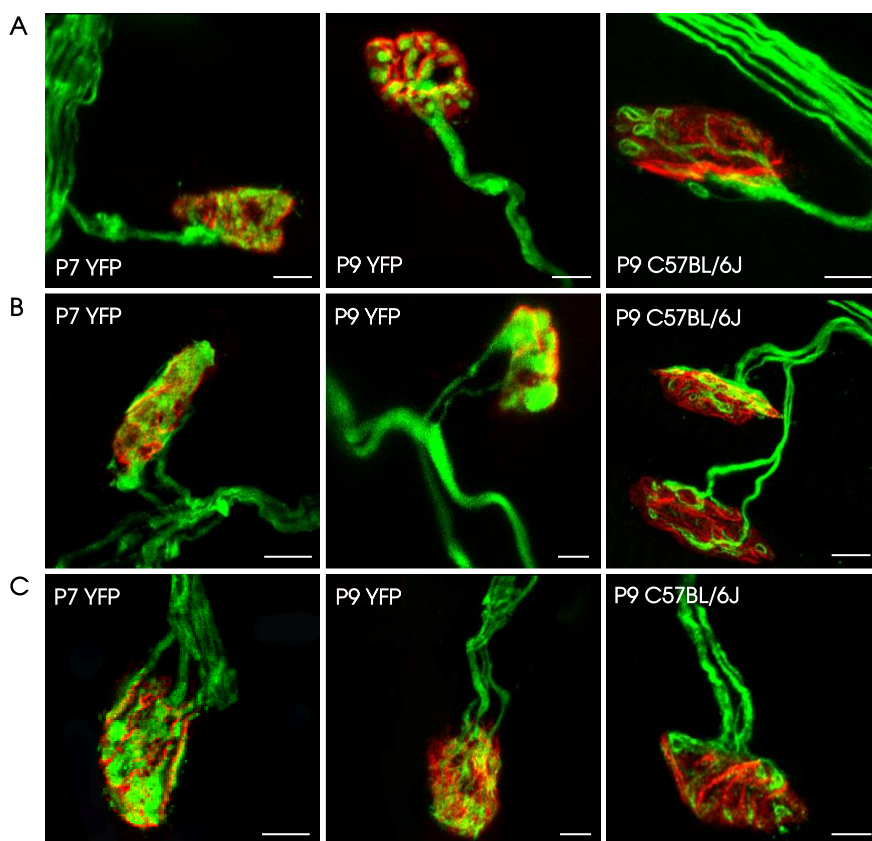


Figure 30. Confocal immunofluorescence images. The picture shows some representative confocal immunofluorescence images of one axon (A), two axons (B) and three or more (C) from YFP and C57BL/6J mice. Scale bar: 10 µm.

1.2.1.2. Stimulation of the mAChRs. Effect of oxotremorine

Figure 31 shows the percentage of singly-, doubly- and triply (or more) innervated NMJs in the untreated YFP control mice and after 2 (P7), 4 (P9) and in some cases 10 (P15) applications (one application every day after P5) of the mAChR agonist oxotremorine (OXO) and the antagonists atropine (AT), pirenzepine (PIR), methoctramine (MET) and muscarinic toxin 3 (MT3). The potent and well characterized unselective agonist OXO was used. Two subcutaneous applications (at P5 and P6) on the YFP LAL muscle surface results in a significant acceleration at P7 of the axonal elimination process (**figure 31A**; n=820 NMJs, N= 4 mice), because of the increase in monoinnervated NMJs ($p<0.005$) and the reduction in dual ($p<0.05$) synapses. It seems that the muscarinic mechanism, when stimulated, accelerates the axonal elimination rate and transition to the monoinnervation state.

However, four applications (P5-P8) of OXO (**figure 31B**; n=865 NMJs, N= 4 mice) do not lead to any significant change at P9 ($p>0,05$). This indicates that the effect of muscarinic stimulation diminishes and tends to peak close to the normal values of axonal elimination around four days after stimulation has begun. Therefore, there is a window around P5-P6 in which mAChR can be forced to accelerate synapse elimination. However, exogenous stimulation with the agonist only reveals that muscarinic signaling has the potential to accelerate postnatal axonal disconnection but does not explain what the tonic muscarinic control is like in a normal situation. Therefore, it has been investigated how blocking the M_1, M_2 and M_4 mAChR subtypes *in toto* or selectively (those subtypes) observed in functional developing NMJ, (Santafé et al., 2003, 2004, 2008; Garcia et al., 2005) can affect synapse elimination.

1.2.1.3. Unselective inhibition of mAChRs. Effect of atropine

Figure 31A shows that two subcutaneous applications of AT (at P5 and P6) in the YFP LAL muscles analyzed at P7 significantly reduce the percentage of triple junctions, increase the percentage of dual junctions and have no effect on the percentage of single junctions (Control PBS (n=1533 NMJs, N= 6 mice): 1 axon: 22.69% ± 1.04%; 2 axons: 50.20% ± 2.75%; 3 or more axons: 27.11% ± 3.18%. AT application (n=1343 NMJs, N= 3 mice): 1 axon: 23.53% ± 3.18% ($p>0,05$); 2 axons: 60.78% ± 2.36% ($p<0,01$); 3 or more axons: 15.69% ± 0.79% ($p<0,05$)).

Thus, the rate of transition from three to two speeds up but the overall elimination process does not continue to the point of significantly increasing monoinnervation. This indicates that AT has a dual effect: namely, it increases axon loss in triple junctions and reduces loss in double NMJs. It seems that NMJs or nerve terminals of different levels of maturity have different sensitivities and respond differently to this potent muscarinic pan-inhibitor.

Daily AT applications between P5 and P8 lead to a significant retardation of axonal elimination at P9 (**figure 31B**; n=1032 NMJs, N= 4 mice) with persistent polyinnervation due to the higher percentage of dual junctions ($p<0,005$) the corresponding decrease in monoinnervated synapses ($p<0,005$) and an almost normal number of triple junctions ($p>0,05$). This clearly indicates that blocking the mAChR can persistently obstruct the two-to-one transition of the elimination process. However, unlike the OXO effect (which tends to disappear at P9 after accelerating elimination at P7), the effect of AT seems to be maintained throughout the period P5-P9 at least in relation to the two-to-one transition. It seems, then, that in normal conditions, the presynaptic muscarinic mechanism increases the rate of axonal loss at least in dual junctions in the period P5-P9 and that this effect can be increased at P7 by using an exogenous agonist.

1.2.1.4. Selective block of the mAChRs

Previously, it has been shown that unselective inhibition of mAChRs studied by AT induced an acceleration of the transition three-to-two axons at P7 and a retardation of axonal elimination at P9. How are the various mAChR subtypes that operate in the postnatal NMJ (M_1, M_2 and M_4) involved individually in axonal elimination? It has been selectively blocked M_1 (PIR), M_2 (MET) and M_4 (MT3) and observed the NMJ at P7 (daily applications on the LAL surface at P5 and P6, **figure 31A**) and P9 (applications between P5-P8, **figure 31B**). At P7 two subcutaneous PIR applications significantly reduced the percentage of triple ($p<0,005$) and dual junctions ($p<0,05$) and greatly increased the percentage of single junctions ($p<0,005$, n=915 NMJs, N= 4 mice) . Thus, both the three-to-two and the two-to-one rates of transition accelerated considerably and the overall elimination process speeded up. This may indicate that in the normal situation the role of M_1 is to slow elimination down because when it is uncoupled from PIR, the elimination process accelerates. Interestingly, the M_4 blocker MT3 has almost exactly the same effect as the M_1 blocker PIR (n=895 NMJs, N= 4 mice), whereas the M_2 blocker MET does

not have a significant effect at P7 after the two subcutaneous applications ($p>0,05$, n=1012 NMJs, N= 4 mice). As an additional control, in P7 C57BL/6J animals treated with MET the same result was found (Control PBS (n=1533 NMJs, N= 6 mice): 1 axon: 22.69% ± 1.04%; 2 axons: 50.20% ± 2.75%; 3 or more axons: 27.11% ± 3.18%. MET application (n=911 NMJs, N= 3 mice): 1 axon: 22.22% ± 2.56% ($p>0,05$); 2 axons: 50.00% ± 2.74% ($p>0,05$); 3 or more axons: 27.78% ± 2.38% ($p>0,05$)). Thus, at P7 the ensemble M_1/M_4 seems to be involved in a mechanism that delays elimination because when it is blocked the percentage of monoinnervated junctions increased and caused a fast three-to-one transition.

Nevertheless, how can it be explained that at this time (P7) the two-to-one transition is accelerated by the selective blockers PIR and MT3 (and not affected by MET), but that when all mAChR subtypes were blocked with AT this transition was partially delayed? Blocking the whole ensemble of subtypes with AT has a somehow different effect than the individual effects of mAChR subtypes. This apparent contradiction observed with the effects of selective and unselective pharmacological muscarinic inhibitory substances at P7 seems to suggest the existence of other confluent signaling pathways that take part in the process (see below).

However, daily applications of these substances for four days (P5-P8) lead to a much more clearly defined situation at P9 (**figure 31B**). As stated above, four AT applications delay elimination, maintain the number of dual junctions and decrease the number of singly-innervated NMJ, which indicates that the two-to-one transition is slowing down. The same effect (even greater because of the considerable delay in the three-to-two transition) is obtained by blocking M_1 (PIR, $p<0,005$, n=1293 NMJs, N= 3 mice) and M_2 (MET, $p<0,005$, n=976 NMJs, N= 4 mice) but not in this case with the M_4 blocker MT3 ($p>0,05$, n=1177 NMJs, N= 4 mice). As an additional control, in P9 C57BL/6J animals treated with MT3 the same result was demonstrated (Control PBS (n=1352 NMJs, N= 5 mice): 1 axon: 48.17% ± 4.54%; 2 axons: 36.73% ± 2.76%; 3 or more axons: 15.10% ± 4.97%. MT3 applications (n=906 NMJs, N= 4 mice): 1 axon: 51.2% ± 5.77% ($p>0.05$); 2 axons: 39.32% ± 2.53% ($p>0.05$); 3 or more axons: 9.48% ± 2.32% ($p>0.05$)). These data indicate that at this point in the elimination process, both M_1 and M_2 subtypes cooperate in favoring the full sequence of synapse elimination.

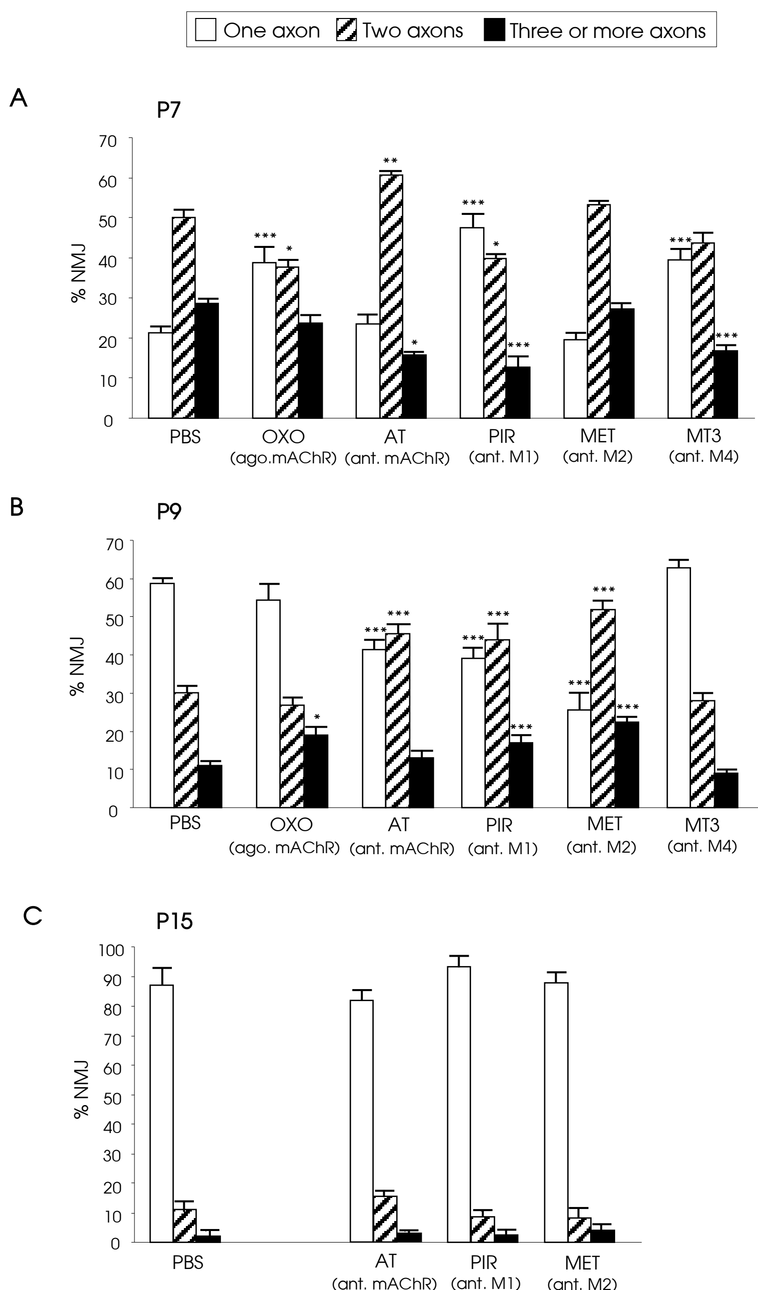


Figure 31. Changes in polyneuronal innervation of the neuromuscular junction after stimulation and inhibition of the mAChRs. The figure shows the percentage of singly-, dually- and triply- (or more) innervated NMJs in the untreated YFP control mice (exposed to PBS applications) and after 2 (P7 in A), 4 (P9, in B) and in some cases 10 (P15, in C) applications (one application every day after P5) of the mAChR agonist oxotremorine (OXO) and such antagonists as atropine (AT), pirenzepine (PIR), methoctramine (MET) and muscarinic toxin 3 (MT3). * $p<0.001$, ** $p<0.01$, *** $p<0.05$.

To investigate the possible persistence of the mAChR effect throughout the period of synapse elimination, daily applications of PIR and MET (the M_1 and M_2 selective antagonists that are effective at modulating axonal elimination at P9) between P5 and P15 (in normal conditions almost 90% of NMJs were monoinnervated at P15) were injected. In spite of the continued presence of selective inhibitors, it has been found that the elimination process came to its normal conclusion by the end of the second postnatal week (**figure 31C**; ($p>0,05$, PIR: n=924 NMJs, N= 3 mice; MET: n=870 NMJs, N= 3 mice)).

This reinforces the suggestion that several signaling mechanisms between the endings in competition cooperate (and substitute each other) to resolve the correct synaptic connection in a multifactorial process.

1.2.1.5. Other signaling mechanisms involved in axonal loss

Several signaling pathways connect the cells that make synapses thus involving of different types molecules and receptors could be presented in the axonal loss process. Previously, the existence of a mechanism based on mAChR coupled to PKC and VDCC was indicated. However, there were other types of receptors that could be implicated. Here, it has been studied the possible involvement of adenosine receptors and neurotrophin receptors (here the representative TrkB receptor for BDNF and NT4) in the complex period of axonal elimination around P7-P9 (**figure 32**).

To the LAL muscle, it has been subcutaneously applied the AR inhibitor 8SPT, the AR agonist ADO and the TrkB blocking pathway agent TrkB-Fc to sequester endogenous BDNF/NT4 neurotrophins. With the 8SPT (n=920 NMJs, N= 4 mice) and TrkB-Fc (n=1113 NMJs, N= 4 mice) blockers at P7 it has been observed a clear acceleration in the three-to-two rate (8SPT: $p<0,005$; TrkB-Fc: $p<0,05$) that was very similar to the acceleration in the two-to-one rate. These substances accelerate axonal elimination on the NMJ and, therefore, the physiological role in normal conditions of the AR and TrkB pathways at P7 seems to delay the axonal loss process. This is confirmed for the AR because exposure to the physiological agonist ADO results in a significantly higher number of triple junctions and a significant reduction in the number of dual junctions ($p<0,005$, n=923 NMJs, N= 4 mice).

This indicates an ADO-induced retardation of axonal elimination. Which AR subtypes are involved in the ADO effect? It has been analyzed axonal elimination after selectively blocking A_1 with DPCPX (n=1160 NMJs, N= 4 mice) or A_{2A} inhibition with SCH-58261(n=963 NMJs, N= 4 mice) (**figure 32A**). The data show that axonal loss (the full three-to-one transition) is accelerated by both inhibitors ($p<0{,}005$), which indicates that in normal conditions without inhibition both A_1 and A_{2A} are associated with delaying loss.

Interestingly, at P9, neurotrophin signaling seems to reverse their coupling to the axonal loss process because TrkB-Fc (acting between P5-P8) considerably delays elimination (resulting in more dual and fewer monoinnervated NMJ; $p<0{,}005$, n=863 NMJs, N= 4 mice), which indicates that in a normal situation BDNF/NT4 mediators change their role and accelerate elimination, as has been described above for the muscarinic mechanism.

At P9, the purinergic mechanism also seems to tonically accelerate axonal elimination to the maximum rate because the AR pan-inhibitor 8SPT delays the process (an effect of the A_1 and A_{2A}, **figure 32B**; n>900 NMJs, N= 4 mice in each case) with no effect of the agonist ADO ($p>0{,}05$, n=908 NMJs, N= 4 mice).

Therefore, it seems that AR may behave biphasically in the critical period between 5-9 postnatal days. An initial delay in axonal loss at P7 (an A_1 and A_{2A} mediated effect which can be reinforced by exogenously added ADO) is followed by an A_1 and A_{2A} mediated tonic acceleration at P9. To sum up, the two receptor sets (TrkB and AR) initially delay (P7) axonal loss but promote axonal disconnection at the beginning of the second postnatal week (P9) as mAChRs do.

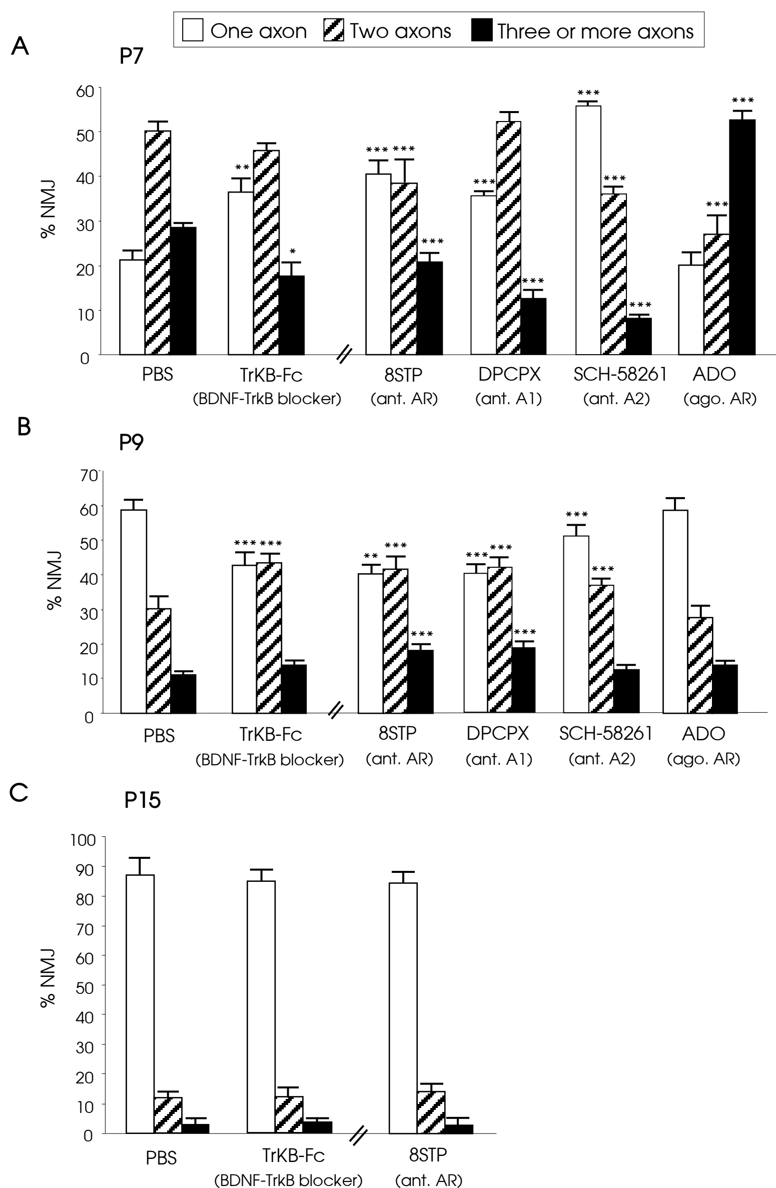


Figure 32. Involvement of ARs and TrkB receptor in axonal elimination. The figure shows the percentage of the singly-, dually- and triply- (or more) innervated NMJs in the YFP control mice exposed to PBS, and after 2 (P7 in A) and 4 (P9, in B) applications (one application every day after P5) of the TrkB blocking pathway agent TrkB-Fc to sequester endogenous BDNF/NT4 neurotrophins and the AR pan-inhibitor 8SPT and the AR agonist ADO. It has been also studied axonal elimination after selectively blocking A_1 with DPCPX and inhibiting A_{2A} with SCH-58261. The control for these selective inhibitors was PBS + DMSO (not shown in the figure) which shows no differences from PBS used as a control by itself. * $p<0.001$, ** $p<0.01$, *** $p<0.05$.

1.2.2. POSTSYNAPTIC RECEPTORS CLUSTER DURING POSTNATAL MATURATION

It has been analyzed the morphological maturation of the postsynaptic apparatus in the same experimental conditions as those in the previous study on axon loss. The axonal elimination process was accompanied by changes in the morphology of the nAChR clusters in the postsynaptic component. On the basis of criteria from previous studies on developing mammalian NMJs (Steinbach, 1981; Slater, 1982a, 1982b; Bewick et al., 1996; Lanuza et al., 2002; Garcia et al., 2011), the following maturation stages (MS1–MS4) were defined (**figure 33**). As normal maturation takes place, changes in the nAChR distribution transform the uniform nAChR oval plaque with an indistinct boundary seen at birth (MS1) into an elongated plaque with a few hints of heterogeneities in receptor density (MS2). This then changes into clusters with small areas of low nAChR density appearing as holes (MS3) that were not innervated. The oval nAChR areas have been transformed into a more mature branched pattern with a moderately convoluted external border and high and low receptor density areas (MS4). The edge of the holes usually has a high density of receptors.

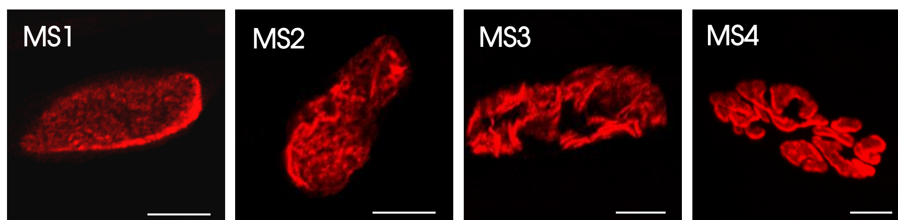


Figure 33. Morphological maturation changes of nicotinic acetylcholine receptors (nAChR). The axonal elimination process is accompanied by changes in the morphology of the nAChR clusters in the postsynaptic membrane. The following maturation stages (MS1–MS4) were defined. MS1: Uniform nAChR oval plaque with an indistinct boundary seen in the majority of NMJs at birth. A uniformly distributed porosity can be observed within this plaque. MS2: nAChR elongated oval plaque with a few hints of inhomogeneities in receptor density. The nAChRs are denser on a few narrow ridges that occur within the plaque. MS3: An oval nAChR plaque with one or more fluorescence-free 'holes'. These holes are not innervated. MS4: The oval nAChR areas have been transformed into a more mature branched pattern with a moderately convoluted external border and high and low receptor density areas. The edge of the holes usually has a high density of receptors. Scale bar: 10 µm.

In **figure 34**, it has represented the percentages of the MS1-MS4 nAChR clusters plotted at days P5-P15 according to the nAChRs morphology described in **figure 33**. At P5, most of the receptors (78,18%) were in MS1 while 20,52% were in MS2 and only 1,30% were in MS3. At P7, as the maturation advance, the number of MS1 receptors decrease significantly and the largest number of the receptors (87,40%) were in MS2. At P9, the number of MS2 decrease (74,36%) in order to increase the number of receptors in MS3 (20,34%). At P10, the values were quite similar as P9 since it was only one day later. At P15, the major number of receptors were in MS4 (77,55%) and few receptors in MS3 (19%).

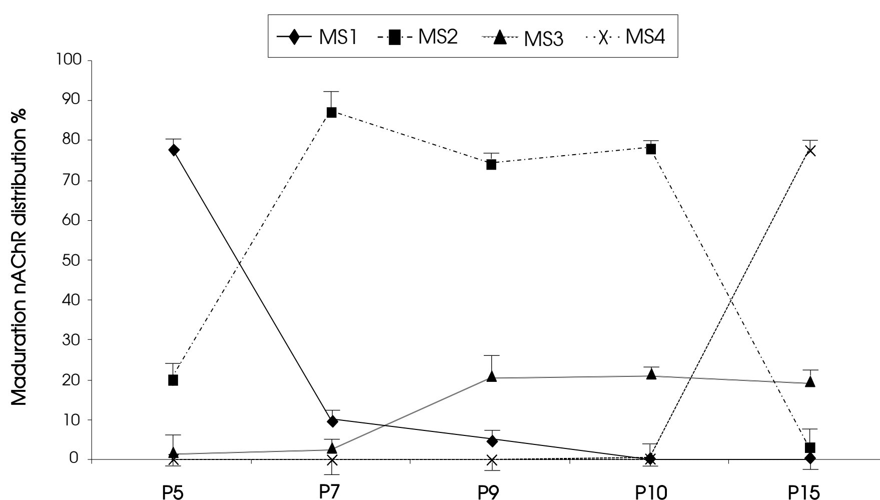


Figure 34. Postnatal morphological maturation of the postsynaptic apparatus. The graph shows the percentages of the MS1-MS4 nAChRs clusters plotted in the interval P5-P15 days. At P5, the majority of the nAChRs clusters are in maturation stage 1 (MS1). At P7 and P9, as the maturation advance, the number of nAChRs in MS1 decrease while the number of nAChRs in maturation stage 2 (MS2) and maturation stage 3 (MS3) increase. At P15, the larger part of nAChRs are in maturation stage 4 (MS4).

1.2.2.1. mAChRs influence on the postsynaptic maturation

On the other hand, it has been studied the agonism and antagonism of mAChRs on the postsynaptic component and the receptors were classified following the four maturation stages (MS1, MS2, MS3 and MS4) describe above.

Figure 35 shows the percentage of MS1-MS4 clusters in the NMJ of the untreated YFP control mice (PBS) and after 2 (P7, **figure 35A**), 4 (P9, **figure 35B**) and 10 (P15, **figure 35C**) applications of the muscarinic substances considered. After the mAChR antagonists AT, PIR and MET (MT3 does not unambiguously modify the postsynaptic clusters) had been applied for two days, at P7 it has been found changes in the morphological maturation of the postsynaptic apparatus. Generally there was a high percentage of differentiated MS3 clusters ($p<0,005$) and fewer MS1 and MS2 ($p<0,005$; **figure 35A**). The fact that postsynaptic maturation accelerates after muscarinic inhibition supports the notion that in normal conditions (without inhibition) the M_1 and M_2 subtypes have a tonic role and delay maturation. Because OXO does not have a definite significant effect ($p>0,05$; **figure 35A**), the tonic muscarinic effect may operate at close to its maximum rate.

At P9, the selective muscarinic drugs PIR, MET and MT3 accelerated cluster maturation, and produced fewer MS1 and more MS3 clusters (for MS3: PIR ($p<0,005$); MET i MT3 ($p<0,05$); even MS4 for MT3; see **figure 35B**). This also indicates that at P9 the M_1, M_2 and M_4 subtypes are involved in delaying the normal maturation process in normal conditions. However, AT does not change the normal percentage of the cluster subtypes (though the MS3 subtype is also innervated by 2–3 axons, $p>0,05$) and OXO moderately accelerates maturation (by reducing MS1 ($p<0,005$) and increasing the MS2 subtype, $p<0,01$). Thus, the use of the subtypeunselective drugs AT and OXO reveal the complex involvement of the mAChRs in the morphological maturation process of the postsynaptic receptor clusters. The coincident contribution of other signaling will be considered below.

At P15, the selective M_1 and M_2 muscarinic inhibitors, and especially with PIR, it has been observed that postsynaptic maturation seems to be slower and partially retained at the MS3 subtype ($p<0,005$; **figure 35C**).

Thus, as far as postsynaptic clusters are concerned, in normal conditions mAChRs tend to produce some delay in maturation throughout the P5-P9 period and this effect is extended at P15 when axonal elimination is almost complete whether muscarinic modulators are used or not.

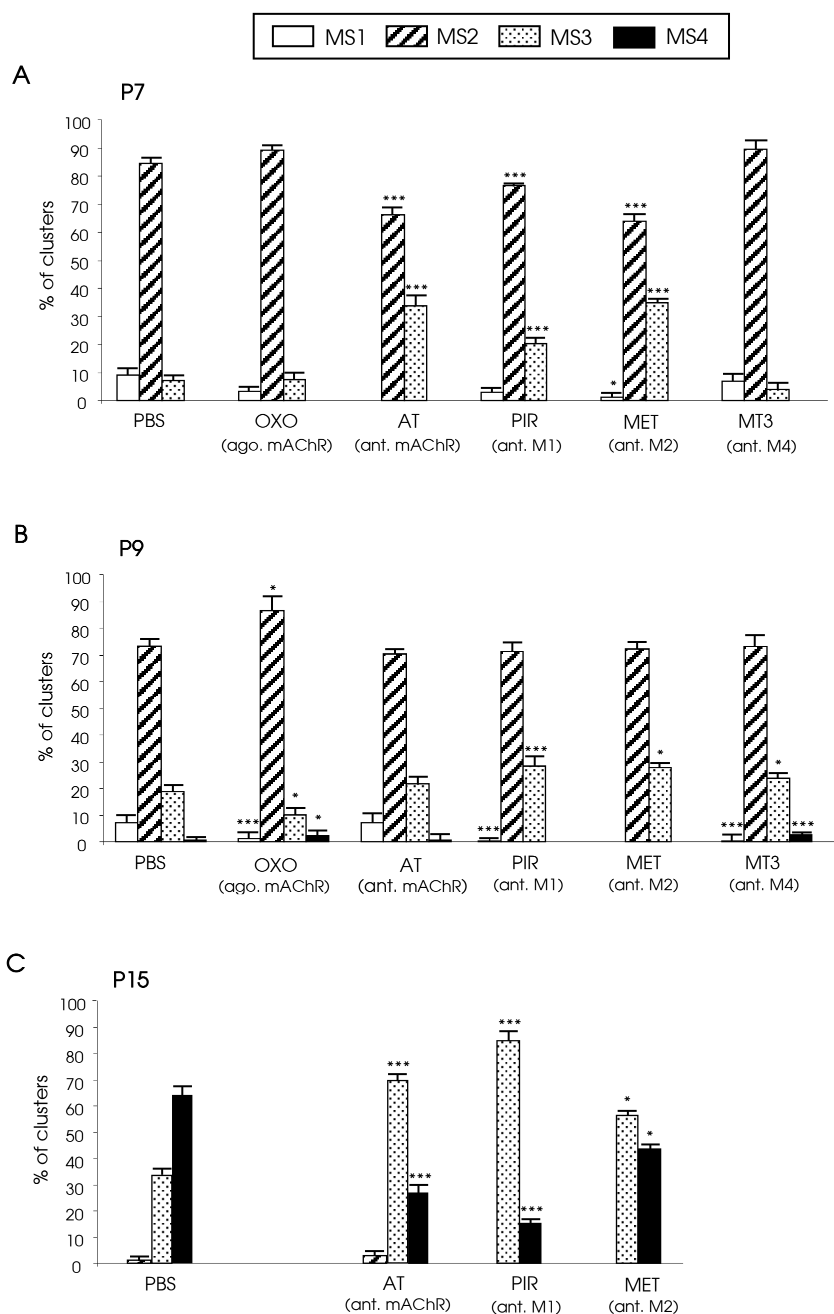


Figure 35. Maturation of postsynaptic nicotinic acetylcholine receptor (nAChR) clusters after stimulation and inhibition of mAChRs. Percentage of MS1-MS4 clusters in the NMJ of untreated YFP control mice (exposed to PBS), and after 2 (P7, in A), 4 (P9, in B) and 10 (P15 in C) applications of the muscarinic substances considered: OXO, AT, PIR, MET and MT3. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

Figures 36A, B and **C** also show the percentage of MS3 clusters (postsynaptic clusters in advanced morpholocical maturation) with one, two or three (or more) axons for each day. This percentage can be taken as an indication of the correspondence between pre- and postsynaptic maturation.

At P7, (**figure 36A**) AT, PIR and MET increased significantly the number of MS3 multiinnervated. For instance, AT increase two times the MS3 innervated by two axons respect PBS (p<0,005). The number of NMJs innervated by one axon in MS3 clusters decreased in AT (p<0,005), PIR (p<0,05) and MET (p<0,005) experiments.

At P9, (**figure 36B**) many MS3 (for AT, PIR and MET experiments) were innervated by 3 axons. PIR and MET antagonists (p<0,005) and AT antagonist (p<0,05) induced that a large number of NMJs still innervated by three axons while the postsynaptic apparatus was maturating and becoming in MS3. Moreover, AT, PIR and MET experiments decrease the number of MS3 innvervated by one axon (p<0,005). However, in MT3 experiments the number of MS3 clusters does not have a definite significant effect (p>0,05).

At P15 (**figure 36C**), most MS3 are already monoinnervated in the presence of PIR (p<0,01) and MET and AT whereas some of these MS3 are dually-inntervated in the presence of PIR (p<0,01) and MET (p<0,05).

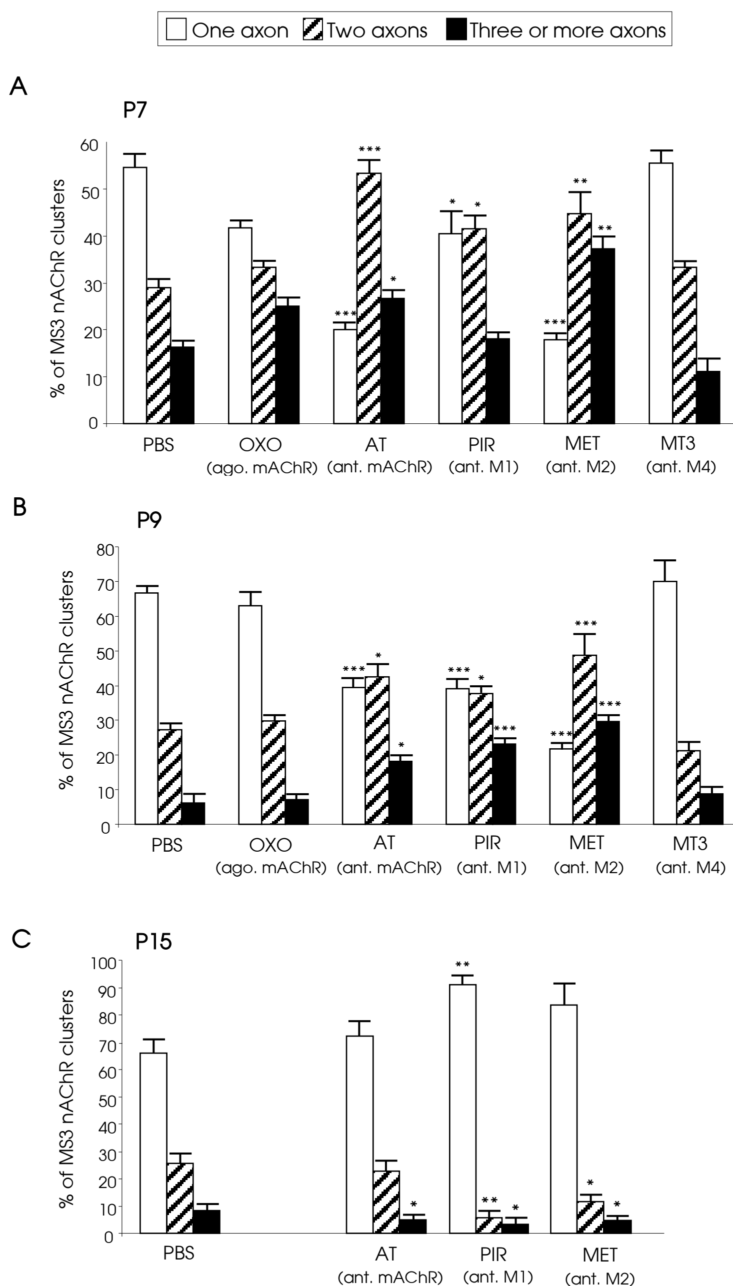


Figure 36. Correspondence of pre- and postsynaptic maturation in the MS3 clusters after stimulation and inhibition of the mAChRs. For each day considered (P7 in A, P9 in B and P15 in C) the figure shows the percentage of MS3 clusters (the oval nAChR plaques with fluorescence-free holes that mature at a faster rate) with one, two and three or more axons as an indication of the appropriate correspondence of the pre- and postsynaptic maturation. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

As is shown in **figure 36**, many MS3 (for AT, PIR and MET experiments) are innervated by 2-3 axons as they were at P7. Thus, a disconnection between the maturation of the presynaptic and postsynaptic component is appreciated. In LAL muscle which were injected with MET (M_2 antagonist), it has been observed that many of MS3 clusters were still innervated by 2-3 axons (**figure 37**). Thus, polyinnervated axons are in MS3, meaning an acceleration of the postsynaptic maturation. This phenomenon could indicated that there is some imbalance in the appropriate pre- and postsynaptic maturation. The **figure 37** shows two examples of a still polyinnervated almost mature MS3 cluster from an YFP P7 muscle.

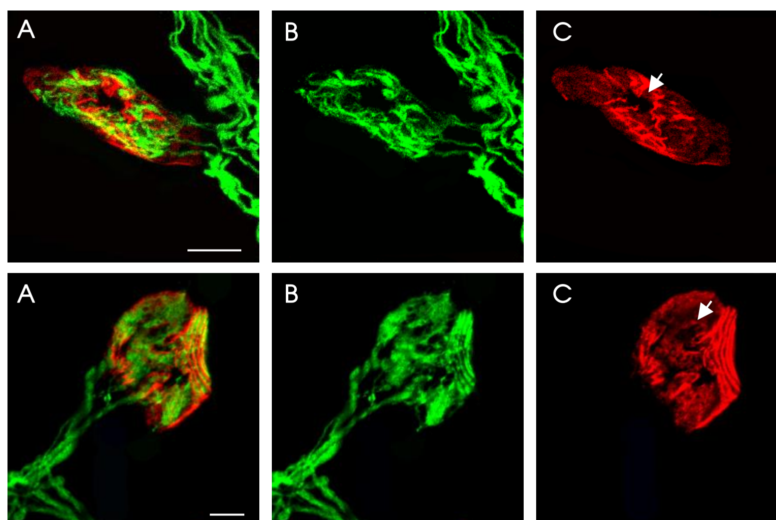


Figure 37. Examples of polyinnervation in MS3 clusters from an YFP P7 muscle. The pictures show two examples of a still polyinnervated almost mature cluster. **A**. The merge of the YFP and α-BTX-TRICT. **B.** The YFP label. **C.** α-BTX-TRICT label exhibits the disposition of nicotinic acetylcholine receptors (nAChRs). The flashes indicate the presence of holes indicating that the receptors are in maturation stage 3 (MS3). Scale bar: 10 μm.

1.2.2.2. Other signaling mechanisms involved in postsynaptic maturation

Figure 38A is shown that after two days of using TrkB-Fc to sequester endogenous BDNF/NT4, nAChR maturation was delayed at P7 because of the persistence of many MS1 clusters ($p<0{,}005$). This indicates that the normal stimulation of the TrkB pathway promoted postsynaptic maturation at around P7. This tendency was reversed at P9 after four days of exposure to TrkB-Fc because of the clear increase in the MS3 subtype with respect to the untreated control ($p<0{,}05$; **figure 38B**).

With regard to the AR pathway, at P7 it has been found that the unselective antagonist 8SPT had no effect on the maturation of postsynaptic clusters ($p>0{,}05$; **figure 38A**) although when 8SPT was applied in the period P5-P8 (observation at P9, **figure 38B**) MS2 clusters increased and MS1 and MS3 clusters decreased, which indicates some delay in the transition from MS2 to MS3. Thus, AR in normal conditions without inhibition can accelerate maturation somewhat during the P7-P9 period. Interestingly, exposure of the LAL muscle to the agonist ADO does not unambiguously change the normal distribution of the clusters at P7 (although it decreases MS3 slightly and a number of these clusters are innervated by three or more axons ($p<0{,}01$; **figures 38A** and **39A**) and P9 (although there is a slight decrease in MS1; $p<0{,}01$). This indicates that the tonic effect of the AR manifested by using 8SPT can not be clearly changed with exogenously added agonist. Which AR subtypes are involved in the tonic effect of endogenous ADO? It has been analysed the maturation of nAChR clusters after selective block of A_1 with DPCPX or A_{2A} block with SCH-58261. Our data indicate that blocking A_1 at P7 and both A_1 and A_{2A} at P9 delays the maturation of normal clusters meaning that both receptor subtypes can accelerate postsynaptic maturation in normal conditions.

Ten applications (one application every day after P5) of TrkB-Fc reveal some delay of the postsynaptic maturation at P15 (increased MS3 and less MS4 clusters, **figure 38C**). Thus, the TrkB pathway seems to have a complex effect on postsynaptic maturation (accelerated at P7, delayed at P9 and accelerated once again thereafter).

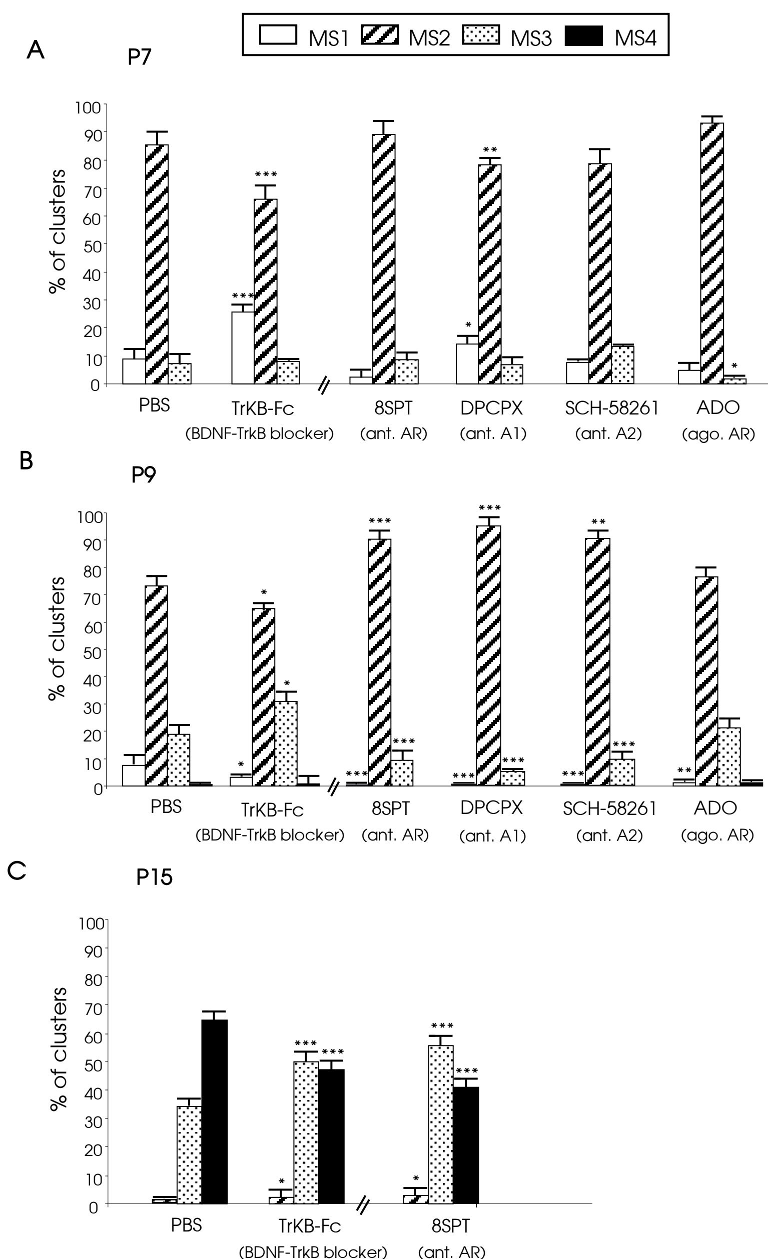


Figure 38. Involvement of the ARs and TrkB receptor in the morphological maturation of the postsynaptic apparatus. The figure shows the percentage of the MS1-MS4 clusters in the NMJ of the untreated YFP control mice (exposed to PBS), and after 2 (P7 in A) and 4 (P9, in B) applications (one application every day after P5) of the TrkB blocking chimera TrkB-Fc, the AR pan-inhibitor 8SPT and the AR agonist ADO. Axonal elimination after selectively blocking A_1 with DPCPX and inhibiting A_{2A} with SCH-58261 were studied. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

Figures 39A, B and **C** is also shown the percentage of MS3 clusters (postsynaptic clusters in advanced morpholocical maturation) with one, two or three (or more) axons for each day. This percentage can be taken as an indication of the correspondence between pre- and postsynaptic maturation.

Figures 39 shows the percentage of MS3 clusters after stimulation and inhibition of the ARs by 8SPT and ADO and the selective antagonists of adenosine subtypes receptors: DPCPX and SCH-58261. On the other hand, the percentage of MS3 clusters were tested after inhibition of TrkB pathway by the antagonist TrkB-Fc chimera.

Concerning AR, many of these MS3 clusters are polyinnervated at P7 (**figure 39A**). For instance, DPCPX experiment was shown a large number of MS3 clusters innervated by two axons ($p<0,005$). However, only 8SPT experiments remain polyinnervated with two or three axons at P9 ($p<0,005$; **figure 39B**). At P15, muscarinic 8SPT antagonist had no effect on the maturation of postsynaptic clusters ($p>0,05$; **figure 39C**).

TrkB-Fc chimera had not shown any effect in MS3 clusters at P7 (**figure 39A**). At P9, the number of MS3 clusters innervated by three or more axons increased significantly ($p<0,005$) whereas the number of MS3 clusters innervated by one axon decreased ($p<0,005$). As happened in AR, TrkB-Fc chimera could not modulate the number of MS3 clusters at P15 ($p>0,05$; **figure 39C**).

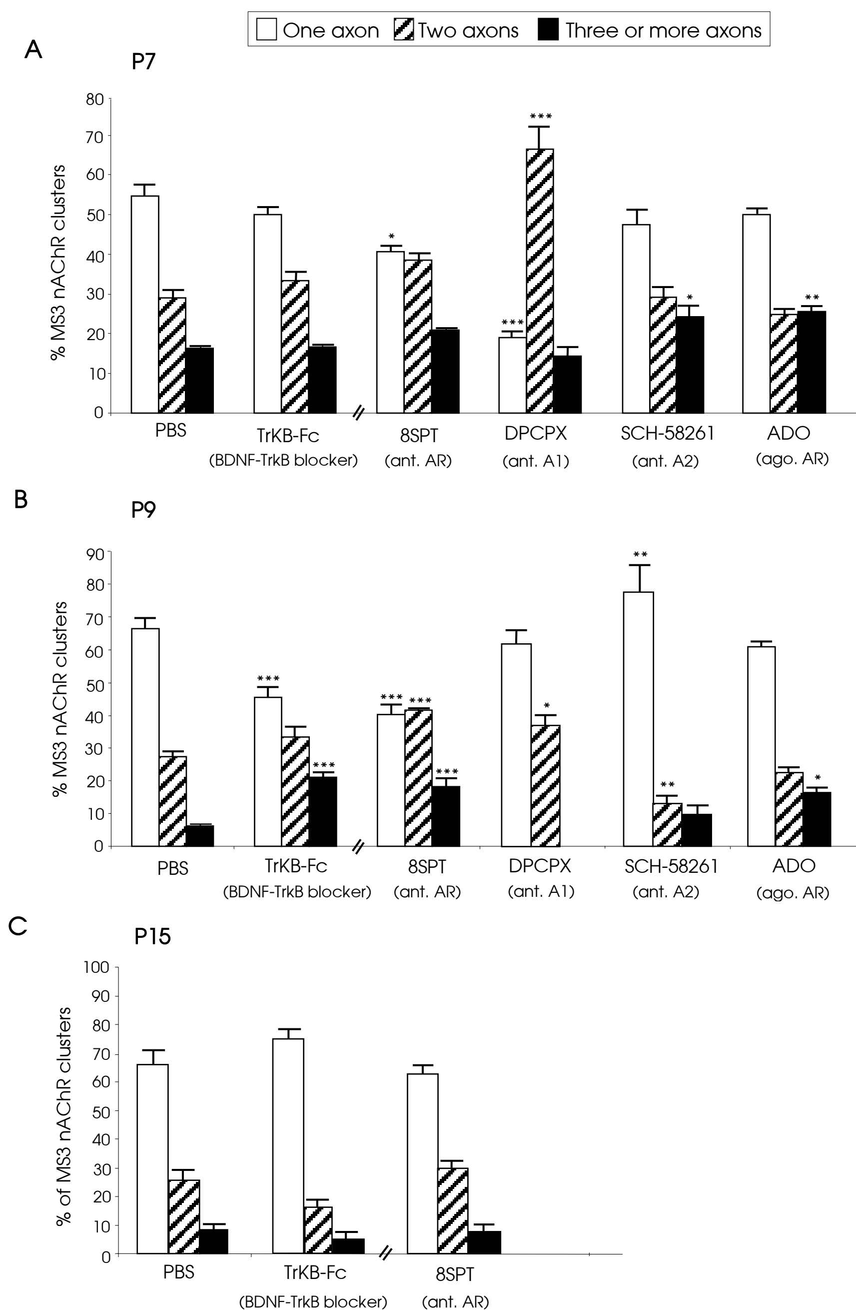


Figure 39. Correspondence of pre- and postsynaptic maturation in the MS3 clusters after stimulation and inhibition of the AR and inhibition of the TrkB receptor pathway. For each day considered (P7 in A, P9 in B) the figure shows the percentage of MS3 clusters with one, two and three or more axons as an indication of the appropiate correspondence of the pre- and postsynaptic maturation. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

The diagram in **figure 40** is a graphic representation for sum up the influence of the mAChRs, and the AR and TrkB receptors on postnatal axonal elimination and postsynaptic maturation.

At P7, mAChRs accelerate the process of synaptic elimination, in concrete, the transition of two axons to one axon. On the postsynaptic site, M_1 and M_2 receptors delay the maturation of the nAChRs. Both adenosine receptors inhibit the synaptic elimination process, for instance, the transition of three axons to one axon. Moreover, A_1 accelerate the potsynaptic maturation. TrkB seems to accelerate the process of the postsynaptic element and delay the process of the presynaptic site (three axons to one).

At P9, M_1 and M_2 accelerate the process otherwise M_1, M_2 and M_4 delay the process of the postsynaptic site. A_1 and A_{2A} accelerate the presynaptic and postsynaptic maturation. Finally, TrkB accelerate the presynaptic monoinnervation and delay the postsynaptic nAChRs maturation.

In summary, TrkB and AR delay axonal elimination at P7 and promote axonal disconnection at the beginning of the second postnatal week (P9) as mAChRs do.

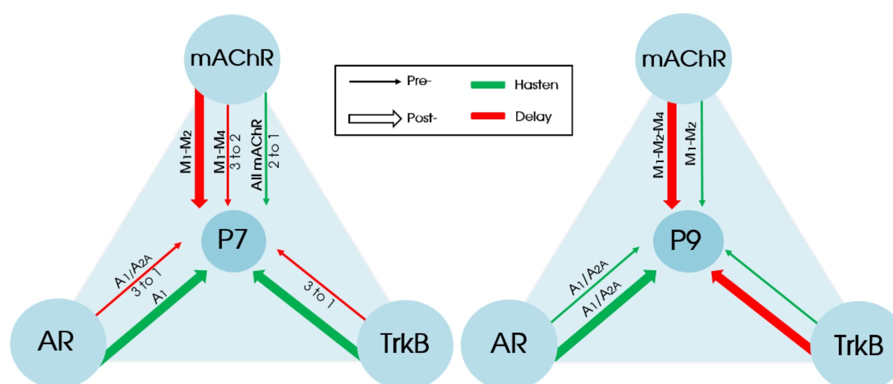


Figure 40. Graphic representation of the influence of the mAChRs, ARs and TrkB receptor on postnatal axonal elimination and synaptic maturation. On the left of the image there are the summary of the results at P7 and on the right of the image at P9. The thin line indicates the presynaptic effect whereas the thick line the postsynaptic effect. Green means a hasten in the synaptic elimination process and red a delay on it.

1.3. DISCUSSION

1.3.1. mAChRs IN THE POSTNATAL ELIMINATION OF NERVE TERMINALS

Although there is not agreement about whether all mAChR subtypes are present in the NMJ (Garcia et al., 2005; Wright et al., 2009), some of these receptors play a role in ACh release both during development (Santafé et al., 2001, 2007a, 2009a) and in the adult (Santafé et al., 2004, 2005). In P6-P7 mice, it has been observed that M_1 and M_4 subtypes are involved in a mechanism that delays axonal elimination. However, the action of all muscarinic receptors as a whole indicates that the muscarinic mechanism increases the rate of axonal loss in dual junctions and, therefore, the final transition to the monoinnervation. It seems that NMJs with different maturation levels have different sensitivities to muscarinic regulation. The relative levels of these receptors or differences in turnover rate may contribute to the different effects observed. Using Western blotting, it has been observed that in the adult, M_1, M_2, M_3 and M_4 receptors are more abundant than in the newborn (Garcia et al., 2005). In fact, changes in the expression of muscarinic receptors during development have been described in embryonic chick heart and retina (McKinnon and Nathanson, 1995), in carotid body, petrosal and superior cervical ganglion of the cat (Bairam et al., 2006) and in rat brain (Tice et al., 1996). In addition, there are differences in the internalization and turnover of the mAChR family members (Reiner and Nathanson, 2012; Ockenga and Tikkanen, 2015) and endocytosis may favor the coupling of the receptors to different signal transduction cascades (Pierce et al., 2001).

However, the M_1-M_2 subtype pair (in substitution of the M_1-M_4 pair) cooperate to favor the full sequence of synapse elimination at P9 (the three-to-one axon transition). The delayed two-to-one transition induced by AT at P7 (which reveals accelerated axonal elimination in normal conditions without the inhibitor) may be interpreted as an early manifestation of the consistent mAChR-modulated axonal loss that is fully manifested at P9. The muscarinic mechanism appears to operate at close to maximum capacity and, therefore, may not be able to increase its efficacy beyond P7 with agonists like OXO. Interestingly, in spite of the continued presence of the M_1 and M_2 inhibitors, the elimination process comes to its normal conclusion at the end of the second postnatal week (P15). This suggests that other signaling mechanisms help to resolve the correct synaptic connectivity. Alternatively, M_1 and M_2 signaling may be not required at all for the final step of axonal elimination because the receptor inhibitors produce only transient

perturbations in elimination but axon loss is completed normally around P15. Our interpretation is that all considered receptors (see later) intervene in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the losers but, thereafter, the time and conditions of the final elimination would occur with some autonomy. In summary, the results show that a tonic muscarinic mechanism initially delays axonal elimination (a selective M_1-M_4 effect). However, the overall mAChR effect may accelerate the last phase of axonal disconnection, the two-to-one transition. Thereafter, the muscarinic effect at around P9 clearly promotes elimination of all supernumerary nerve terminals (an M_1-M_2 effect).

Which mAChR subtypes couple to regulate ACh release? In the mature NMJ, M_1 and M_2 mAChRs modulate evoked transmitter release by positive and negative feedbacks, respectively (Santafé et al., 2003, 2006). M_2 is more prevalent (Wright et al., 2009; Tomàs et al., 2014). During synaptogenesis, (Santafé et al., 2002, 2003, 2004), in the monoinnervated junctions and the strong terminal in dually innervated junctions both M_1 and M_2 are coupled to potentiate ACh release. However, in the weakest nerve terminal in dual junctions only M_2 potentiates release whereas M_1 and M_4 couple to inhibit ACh secretion. A mAChR-PKC-VDCC cascade is involved in controlling ACh release in the weak ending. Blocking PKC, VDCCs (P/Q-, N- or L-type or Ca^{2+} influx) or mAChRs (M_1- and/or M_4-subtypes) can lead to similar percentage increases in the size of the synaptic potentials evoked by weak axons (Santafé et al., 2007b, 2009a; Tomàs et al., 2011).

How are related the release capacity of the strong and weak endings and the loss of axons described here? At P7, the release capacity of the weakest endings was increased by the inhibitors PIR and MT3, whereas ACh release from the strong ending was reduced or unaffected (Santafé et al., 2009a). Thus, the difference in ACh release between the strong and weak nerve endings is reduced, and this fact may change the competitive conditions of the nerve terminals. How is the ACh release capacity of the weak and strong endings in the LAL muscle at P9 were not exactly known. However, between P7 and P9, the percentage of polyinnervated junctions changes only by about 10%. The configuration of mAChR in the monoinnervated synapses is not mature until P15 (Santafé et al., 2003), which suggests that the competitive interactions between axons peak at around P9 and their release capacity is probably not very different from what it is at P7. If this is so, the reduction of the competitive advantage and disadvantage linked to ACh release of the strong and weak endings produced by PIR and the reduction of the

strength of the different axons produced by MET (MT3 does not play at P9) may considerably delay axonal loss.

1.3.2. CONTRIBUTION OF ARs AND TrkB RECEPTOR PATHWAYS

Several data suggest the involvement of other receptors. The mAChR agents alter the time course of the synapse elimination but not its final chronology. Experimental manipulations of the PKC/PKA pathways can also change the time course but not the final conclusion of synapse elimination (Jia et al., 1999; Lanuza et al., 2002; Nelson, 2005; Santafé et al., 2007a). This indicates that different receptors with their intracellular mechanisms can be used in the process of synapse elimination.

ARs are present in the motor terminals of the newborn and adult NMJs (Garcia et al., 2013, 2014). These receptors can collaborate with mAChR to reduce depression during repetitive activity (Correia-de-Sá et al., 1991; Garcia et al., 2013; Santafé et al., 2014). During development, low extracellular concentrations of ADO may activate both A_1 and A_{2A} and have a facilitatory action on ACh release (Pousinha et al., 2010). Our results show that mAChR and AR delay axonal loss at P7 (although mAChR accelerate the last phase of axonal disconnection) but accelerate it at P9. The results showing an additive effect between M_1 and A1 or A_{2A} are an indication of the cooperation between at least these receptors.

The BDNF/TrkB pathway also plays a biphasic role. Judging from the effect of the TrkB-Fc chimera, BDNF initially delays elimination and subsequently accelerates it. Neurotrophins and their receptors in muscle and nerve are expressed in both development and adulthood (Funakoshi et al., 1995; Gonzalez et al., 1999; Ip et al., 2001; Nagano and Suzuki, 2003; Pitts et al., 2006; Garcia et al., 2010f). Low doses of BDNF rapidly induce a TrkB-dependent potentiation at developing NMJs in culture (Poo, 2001). In developing muscles, BDNF increases ACh release in both the weak and strong endings at P6-P7 (Garcia et al., 2010d). In addition, exogenous BDNF increases the percentage of functional polyinnervated junctions (Tomàs et al., 2011). Interestingly, exogenous BDNF infusion delayed synapse elimination in the mouse LAL muscle (Je et al., 2013). The delaying effect of the TrkB pathway on axonal elimination at P7 described here may be related with the BDNF potentiation of the weakest endings about to be eliminated. However, blocking the TrkB receptor or neutralizing endogenous BDNF with the TrkB-

Fc chimera at P7 does not affect the quantal content of the weak endings but increases release in the strong ending, which suggests that endogenous BDNF, in this developmental period, may surprisingly reduce release in the strongest ending (Garcia et al., 2010d). The delaying effect of the TrkB pathway on axonal elimination at P7 may be related to the BDNF-mediated lesser release and presumed lesser competitive force of the strong axon. The TrkB pathway accelerates elimination at P9. The progressive maturation of the NMJ at P9 may change the operation conditions of the BDNF/TrkB pathway in the strongest endings resulting in more efficient competition and axonal elimination (Mantilla et al., 2004; Garcia et al., 2010d).

1.3.3. INVOLVEMENT OF THE mAChRs, ARs AND TrkB RECEPTOR IN THE MATURATION OF nAChR CLUSTERS

mAChRs (Garcia et al., 2005), ARs (Garcia et al., 2013) and TrkB receptors (Garcia et al., 2010e) are present in the postsynaptic site of NMJs and are involved in organizing them (Gonzalez et al., 1999; Belluardo et al., 2001; Loeb et al., 2002; Peng et al., 2003). The changes that it has been observed may be caused by the pharmacological tools directly acting on these receptors, as a side-effect of a primary effect on the axonal elimination rate or a combination of the two mechanisms. The first change in synapse elimination may be a reduction in the quantal efficacy because of a local decrease in nAChR density (Colman et al., 1997). This postsynaptic change may begin before the overlying axon withdraws (Balice-Gordon and Lichtman, 1993). However, polyneuronal innervation decreases considerably at a time when relatively few postsynaptic nAChRs are lost (Lanuza et al., 2002; Nelson et al., 2003). It has been found that several situations of increased axonal loss or retention did not coincide with the maturation of the nAChR clusters, which suggests independent regulation. Interestingly, prolonged M_1 and M_2 inhibition results in a defect in postsynaptic maturation at P15. Especially, M_1 perturbation had a strong effect. This finding suggests a requirement for M_1 and M_2 signaling in postsynaptic maturation and occurs when axon loss has been completed. In addition, AR block with 8SPT and TrkB pathway block with the TrkB-Fc chimera, similarly delay postsynaptic maturation at P15 (in all cases less MS4 mature nAChR clusters) indicating also the need of these signaling pathways in postsynaptic maturation. Selective nAChR-phosphorylation by PKC and PKA is one of the causes of nAChR dispersion and stability, respectively (Nishizaki and

Sumikawa, 1994; Li et al., 2004; Lanuza et al., 2010). An activity-dependent coordinated mAChR-AR-TrkB effect on these postsynaptic kinases could be a key mechanism in NMJ maturation.

In summary, the main observation of the present study is that the coordinated action of the mAChRs (M_1, M_2 and M_4), ARs (A_1 and A_{2A}) and TrkB signaling modulates the conditions of axonal competition and promotes (around P7-P9) the disconnection of supernumerary nerve endings.

CHAPTER 2. Synergistic action of presynaptic muscarinic acetylcholine receptors and adenosine receptors in developmental axonal competition at the neuromuscular junction.

This chapter corresponds to the published article (see Appendix II):

Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Marta Tomàs, Maria A. Lanuza, Victor Cilleros, Josep Tomàs. (2017). Synergistic action of presynaptic muscarinic acetylcholine receptors and adenosine receptors in developmental axonal competition at the neuromuscular junction. Developmental Neuroscience DOI: 10.1159/000458437.

2.1. BRIEF INTRODUCTION AND SCOPE

In the previous chapter (Chapter 1), it has been demonstrated that presynaptic mAChRs (M_1, M_2 and M_4 subtypes), ARs (A_1 and A_{2A}) and TrkB can cooperate in the developmental synapse elimination process at this synapse. Signaling through these receptors seems to be involved in reducing the initial chance (around P7) of eliminating certain weak endings but subsequently increasing (around P9) axonal competition and elimination. However, in spite of the continued presence of the inhibitors of these receptors, monoinnervation is normally achieved at P15. It has been concluded that the three receptor sets intervene in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the losers although a given axon would finally be eliminated with some autonomy and independently of postsynaptic maturation.

In chapter 1, it has been analyzed the role of individual receptors. In this chapter, it has been investigated the additive or occlusive effects of the inhibitors of two of these receptor sets, mAChRs and ARs (the autoreceptors of ACh and adenosine released by nerve endings), and thus the existence of real cooperation between them in synapse elimination at the NMJ.

2.2. RESULTS

2.2.1. INDIVIDUAL ROLE OF PRESYNAPTIC mAChRs AND ARs IN AXON LOSS CONTROL AT P7

The role of these presynaptic mAChRs at P7 was analyzed by using selective inhibitors. The inhibitor action reveals the tonic effect of the receptors in normal conditions. As it has been demonstrated in chapter 1, most of the inhibitors used accelerated transition to monoinnervation in the NMJ and thus accelerated the axonal loss rate.

Figure 41 shows that the inhibitor substances (one application each day between P5-P6) ranged in their ability to promote monoinnervation and reduce the percentage of synapses that were polyinnervated by three or more axons (DPCPX (A_1 subtype inhibitor) < MT3 (M_4 inhibitor) < PIR (M_1 inhibitor) < SCH (SCH-58261 an A_{2A} subtype inhibitor)).

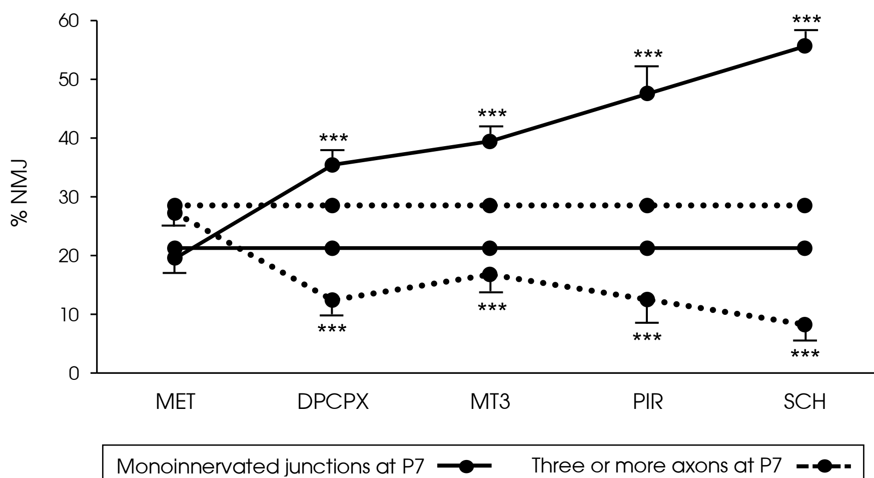


Figure 41. Individual effect of mAChR and AR inhibitors on axon loss at P7. The figure shows the effect of the inhibitors (one application each day between P5-P7) in order of their ability to promote monoinnervation and reduce the percentage of synapses polyinnervated by three or more axons. Only the M_2 blocker MET is unable to significantly change the percentage of monoinnervation. The continuous line represents the monoinnervated junctions; the discontinuous line represents the NMJs innervated by three or more axons. The horizontal lines mark the control values in muscles injected with PBS. *** indicate $p<0.05$.

Only the M_2 blocker MET was unable to significantly change the percentage of monoinnervation, thus showing that it had no apparent effect on axonal loss. The absolute potency of these various receptors in modulating synapse loss cannot be directly assessed and compared because of the difference in the blocking efficacy of the respective selective inhibitors. However, the relative potency of these substances in accelerating axonal elimination suggests that the corresponding inhibited receptor pathway plays a relatively important role in delaying axonal loss.

2.2.2. COOPERATION BETWEEN mAChRs AND ARs AT P7

To determine the possible confluence of these muscarinic and purinergic pathways in the control of axonal loss, it has been investigated here the effect of simultaneous incubation with two inhibitors (two antagonists of two different receptor subtypes, muscarinic, purinergic or both) as a pharmacological tool for revealing the possible occlusive or additive crosstalk effects between the corresponding receptors.

To facilitate comparisons of simultaneously incubations, we represented in the same figures previous data of the individual effect of differents agonists and antagonists. Also **figure 42a** and **figure 43a** show the percentage of innervated NMJs in the untreated YFP P7 control mice (two applications of PBS). **Figure 42** (b-g) shows also the percentage of singly-, doubly- and triply- (or more) innervated NMJs after simultaneous inhibition of two mAChR, a mAChR together with an AR and the two AR together.

For the sake of clarity we have represented in the **figure 42** only the associations between the inhibitors proven (see **figure 41**) to have an individual effect on axon loss (all but MET). The associations of MET with the other substances are represented in **figure 43** (b-e) and it can be seen that there is an unexpected involvement of the M_2 receptors.

In **figure 42**, a superficial interpretation of the complex data suggests that blocking two different receptors simultaneously (dual associations between PIR, MT3, DPCPX and SCH) has roughly the same effect on axonal elimination (on the percentatge of monoinnervation attained at this time) as blocking only one of them. This is true for the associations PIR-SCH, PIR-DPCPX and MT3-SCH. In these

cases, there is no sign that any of these associations have a significantly greater or smaller effect on promoting monoinnervation than the individual effect of the two inhibitors. In fact, the final effect is close to the mean value of the two substances (for instance, see **figure 42e**, when MT3 and SCH act independently the mean percentage of monoinnervated junctions is 47.6% ± 1.25 and when they act simultaneously it is 49.32% ± 4.4, n=1218 NMJs, N= 4 mice p>0.05). Thus, for these associations, there is no additive or occlusive effect and the intracellular pathways of the two receptors seem to converge in a common mechanism fully activated by the action of only one receptor and cannot be increased further or altered by the other receptor.

Interestingly however, when the M_4 blocker MT3 is used in association with the M_1 blocker PIR (**figure 42b**) or the A_1 blocker DPCPX (**figure 42f**), the end result is not significantly different from the individual MT3 effect but differs significantly from the individual effect of PIR (which is greater) and DPCPX (which is smaller). To illustrate this, when PIR act independently the percentage of monoinnervated junctions is 47.56% ± 3.1 and when they act simultaneously with MT3 it is 37.63% ± 1.4, p<0.005, n=1342 NMJs, N= 4 mice, indeed the results are more similar to MT3 applications (39.43% ± 1.6).

In DPCPX happened something similar, when DPCPX act independently the percentage of monoinnervated junctions is 35.46% ± 2.1 and when they act simultaneously with MT3 the percentatge of monoinnervation is 44.43% ± 2.4, p<0.005, n=1497 NMJs, N= 4 mice, indeed the results are more similar to MT3 applications (39.43% ± 1.6). This may mean that M_4 receptors are more prevalent than M_1 and A_1 receptors.

Similarly, when the AR inhibitors DPCPX and SCH act together (**figure 42g**), the result is no different from when DPCPX acts by itself. However, it is significantly different from the individual effect of SCH (p<0.005), which suggests that A_1 has some sort of permissive effect on the A_{2A} pathway.

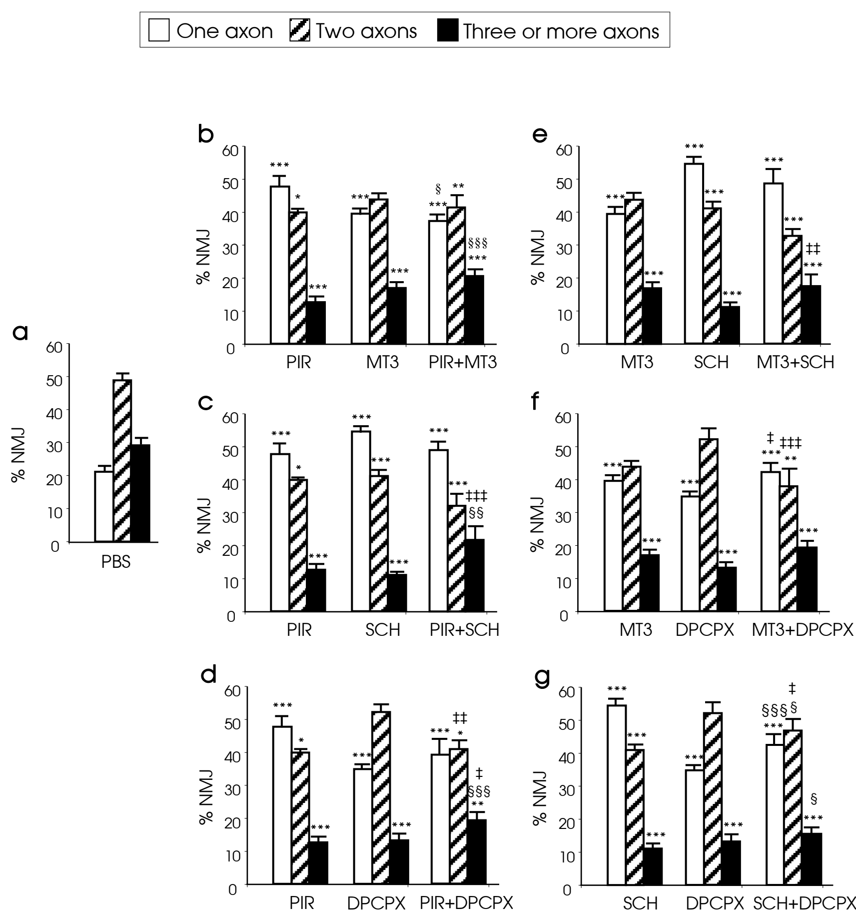


Figure 42. Cooperation between mAChRs and ARs at P7. The figure shows the percentage of singly-, doubly- and triply- (or more) innervated NMJs after simultaneous inhibition of two mAChRs, a mAChR together with an AR and the two ARs together. In this figure, for the sake of clarity, it has been represented only associations between the inhibitors proven to have an individual effect on axon loss (all but MET). The symbols indicate: * $p<0.05$, ** $p<0.01$, *** $p<0.005$ when the correspondent antagonist or combinations of two substances is compared with control PBS. § $p<0.05$, §§ $p<0.01$, §§§ $p<0.005$ when the combination of two substances is compared with the first substance. ‡ $p<0.05$, ‡‡ $p<0.01$, ‡‡‡ $p<0.005$ when the combination of two substances is compared with the second.

As observed in **figure 41**, the M_2 mAChR selective blocker MET does not in itself produce any change in the axonal elimination rate during the period considered. Interestingly, however, when the other inhibitors are applied simultaneously with MET, their individual effects are partially or totally occluded

(**figure 43**). First, a partial occlusion of the SCH and PIR effects is observed (**figure 43b** and **d**). The percentage of the monoinnervated NMJ dropped to roughly the mean value between the MET and the substance considered though this value is still different – higher – than that of the untreated control). Second, the presence of MET totally occludes the MT3 and DPCPX effects (**figure 43c** and **e**). Therefore, MET cancels out the effect of the blockers used to prevent the action of M_4 and A_1 (which are the two receptors that contribute least to delaying axonal loss). However, the powerful effect of SCH and PIR on axon loss cannot be fully prevented, only lessened, by MET.

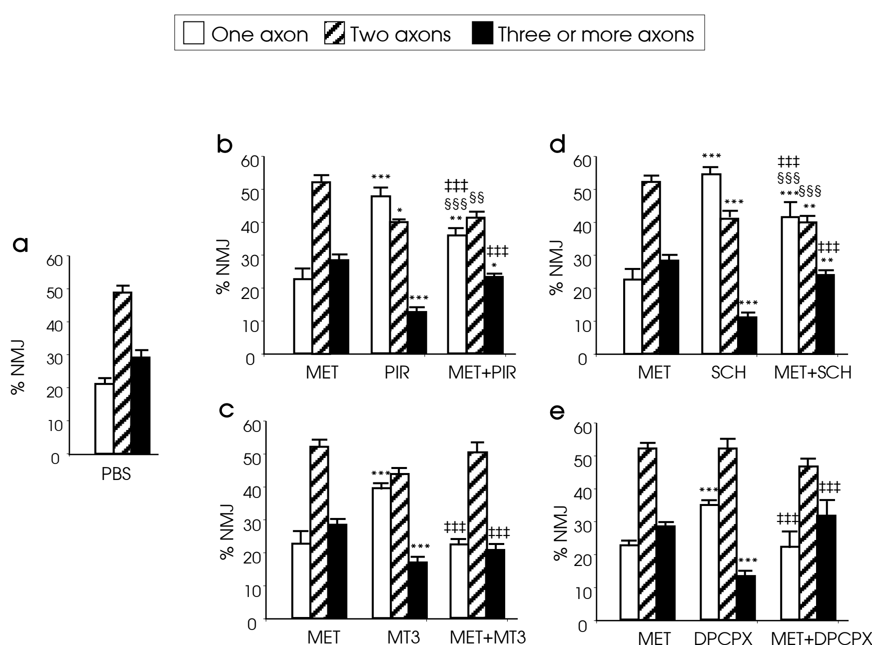


Figure 43. Cooperation between mAChRs and ARs at P7 (only MET with other substances). The shows the percentage of singly-, doubly- and triply- (or more) innervated NMJs after simultaneous inhibition of two mAChRs, a mAChR together with an AR and the two ARs together. In this figure, it has been represented only associations of MET with the other substances. The symbols indicate: * $p<0.05$, ** $p<0.01$, *** $p<0.005$ when the correspondent antagonist or combinations of two substances is compared with control PBS. § $p<0.05$, §§ $p<0.01$, §§§ $p<0.005$ when the combination of two substances is compared with the first substance. ‡ $p<0.05$, ‡‡ $p<0.01$, ‡‡‡ $p<0.005$ when the combination of two substances is compared with the second.

2.2.3. INDIVIDUAL ROLE OF PRESYNAPTIC mAChRs AND ARs IN AXON LOSS CONTROL AT P9

As shown in chapter 1, the effect of the receptor inhibitors (one application each day between P5-P8) delay transition to monoinnervation in the P9 NMJ. However, it is also interesting to remark the effect of the selective inhibitors in order of their ability to finally delay monoinnervation. **Figure 44** shows a high percentage of synapses innervated by three or more axons (MET > PIR = DPCPX > SCH). In this case, only the M_4 blocker MT3 is unable to significantly change the percentage of monoinnervation, which shows that there is no apparent effect on axonal loss at this time. Therefore, the two receptor sets (mAChRs and ARs) finally promote the conditions of axonal disconnection at the beginning of the second postnatal week (P9) (see also in chapter 1).

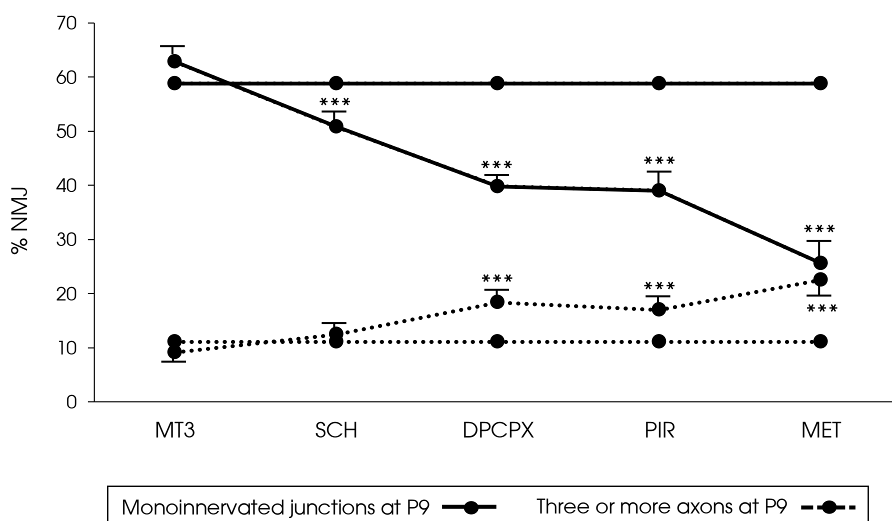


Figure 44. Individual effect of mAChR and AR inhibitors on axon loss at P9. The figure shows the effect of the selective inhibitors (one application each day between P5-P8) in order of their ability to finally delay monoinnervation and keep a high percentage of synapses innervated by three or more axons (MET > PIR = DPCPX > SCH-58261). The M_4 blocker MT3 is unable to significantly change the percentage of monoinnervation in this case. The continuous line represents the monoinnervated junctions; the discontinuous line represents the NMJ innervated by three or more axons. *** $p<0.05$.

2.2.4. COOPERATION BETWEEN mAChRs AND ARs AT P9

As it has been perfomed at P7, **figure 45** and **figure 46** shows the effect of the drug associations applied between P5 and P8 and observed at P9.

The histogram in **figure 45 (a-g)** shows the percentage of singly-, doubly- and triply- (or more) innervated NMJs in the untreated YFP P9 control mice (four applications of PBS) and after four applications of the mAChR and AR selective antagonists and after that the percentage of NMJs after simultaneous inhibition of the two receptors that individually affect axon loss (all the inhibitors but MT3, see **figure 44**).

The associations of MT3 with the other substances are represented in **figure 46 (a-e)** so that the results are more readily understandable.

The data in **figure 45b** show that the association of the mAChR blockers PIR and MET is not the sum of their individual effects and the final result is no different from the individual effect of PIR. Interestingly, however, both AR inhibitors, SCH (**figure 45c**) and DPCPX (**figure 45d**) add their own delaying effect on axonal loss to the delaying effect of PIR for an approximate increase of 58% and 36%, respectively ($p<0.005$).

However, the effect of the two AR blockers is not added to the effect of MET and the result of the dual drug incubation is no different from the MET effect (**figure 45e** and **f**).

When DPCPX and SCH act together (**figure 45g**), the end result is a percentage of monoinnervation that is no different from that given by the control PBS ($p>0.05$), which indicates that both pathways are fully occluded.

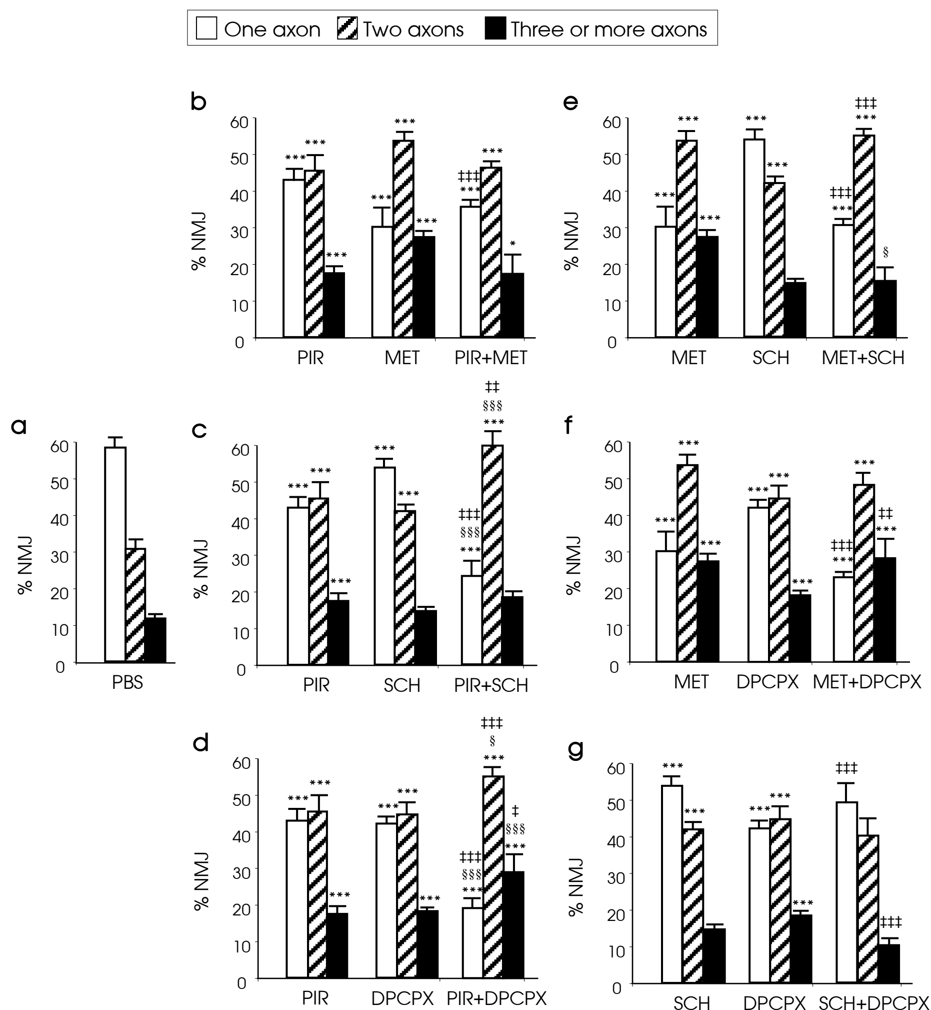


Figure 45. Cooperation between mAChRs and ARs at P9. The data are plotted and shows the percentage of singly-, doubly- and triply- (or more) innervated NMJs in controls (PBS) and after exposure to one inhibitor, after simultaneous inhibition of two receptors that individually affect axon loss (all the inhibitors but MT3). The symbols indicate: * $p<0.05$, ** $p<0.01$, *** $p<0.005$ when the correspondent antagonist or combinations of two substances is compared with control PBS. § $p<0.05$, §§ $p<0.01$, §§§ $p<0.005$ when the combination of two substances is compared with the first substance. ‡ $p<0.05$, ‡‡ $p<0.01$, ‡‡‡ $p<0.005$ when the combination of two substances is compared with the second.

In chapter 1, it has been shown that the M_4 blocker MT3 by itself has no effect on axonal loss at P9. However, **figure 46** shows that the simultaneous application of MT3 with the other muscarinic blockers does not change the effect of PIR (**figure 46b**) though it partly occludes the effect of MET which, however,

continues to significantly delay axon loss (**figure 46c**). Interestingly, MT3 potentiates the delaying effect on axonal loss of both DPCPX and SCH by roughly 33% and 32%, respectively (**figure 46d** and **e**). A representation of these data is shown and discussed in **figure 48**. In some cases, the results with C57BL/6J mice were checked. As an exemple, in P9 C57BL/6J animals treated with MT3 plus MET, it has been found the same result than in YFP animals (C57BL/6J (n=1075 NMJs, N= 3 mice): 1 axon: 42.36 % ± 3.54%, 2 axons: 38.71 % ± 4.97%, 3 or more axons: 18.93% ± 2.18; YFP animals: 1 axon: 48.07 % ± 3.54%, 2 axons: 35.91% ± 4.97%, 3 or more axons: 16.02% ± 2.18. p>0.05). No significant diferences are observed between YFP and C57BL/6J mices treated with PBS.

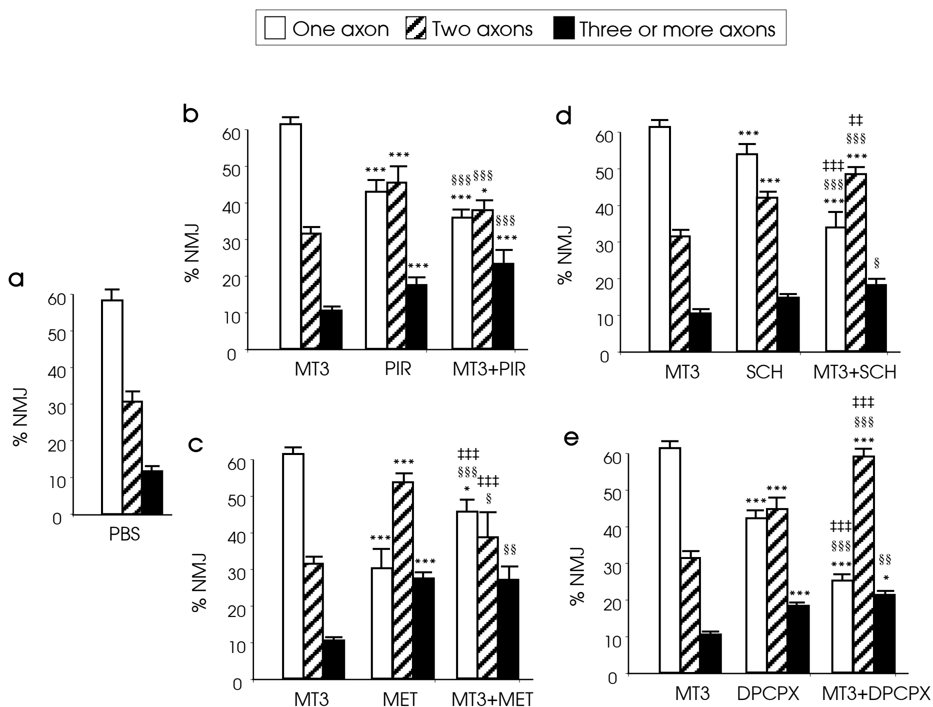


Figure 46. Cooperation between mAChRs and ARs (only MT3 with other substances). The graphic shows the percentage of singly-, doubly- and triply- (or more) innervated NMJs after simultaneous inhibition of two mAChRs, a mAChR together with an AR and the two ARs together. In this figure, it has been represented only associations of MT3 with the other substances. The symbols indicate: * p<0.05, ** p<0.01, *** p<0.005 when the correspondent antagonist or combinations of two substances is compared with control PBS. § p<0.05, §§ p<0.01, §§§ p<0.005 when the combination of two substances is compared with the first substance. ‡ p<0.05, ‡‡ p<0.01, ‡‡‡ p<0.005 when the combination of two substances is compared with the second.

2.3. DISCUSSION

In addition to the main presynaptic neurotransmitter-postsynaptic receptor interaction within a synapse, several signaling pathways coordinate the pre- and post-synaptic cells and associated glia. In the NMJ, the final functional outcome of a synaptic contact is the result of metabotropic receptor-mediated signaling acting on the nerve terminal intracellular protein kinases and modulating voltage-dependent channels and the ready releasable pool of synaptic vesicles, which are the instruments of transmitter release (Takamori, 2012; Tomàs et al., 2014). The ability to release ACh is a decisive factor in the stabilization or loss of motor nerve terminals that are in competition to make synapses during development (Colman et al., 1997; Santafé et al., 2002, 2004, 2007b, 2009a; Buffelli et al., 2003). Postsynaptic-derived trophic substances and glial cells (Lee et al., 2016; Yang et al., 2016) also play a decisive role.

The main finding of the present study (which extends a previous finding in chapter 1) is that the coordinated action and cooperation of mAChRs (M_1, M_2 and M_4 subtypes) and ARs (A_1 and A_{2A}) signaling modulates developmental axonal competition and affects the axonal loss rate. It has been used the term cooperation to define the collaboration between muscarinic and adenosine receptor pathways in developmental axonal loss control, which requires the receptors to work together and act in conjunction. In the present experiments, it has been simultaneously applied two inhibitors (two antagonists of two different receptors) to reveal the possible occlusive or additive crosstalk effects between the corresponding pathways. In previous experiments (chapter 1) two, well defined, developmental periods were observed: P5-P9 and P9-P15. In the first period, it has been noticed the complex involvement of these receptors, which finally resulted in promoting axon loss and accelerating monoinnervation of the NMJ. However, in spite of the continued presence of the inhibitors used, the elimination process finished normally at the end of the second week (P15). Our interpretation is that all the receptors intervene initially in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the losers but, thereafter, the time and conditions of the final elimination occur with some autonomy. Therefore, in the present experiments it has been focused on the P5-P9 period of clear receptor involvement in axonal competition.

As previous data described, mAChR subtypes are present in the NMJ (Garcia et al., 2005; Wright et al., 2009) and some of these receptors play an

important regulatory role in ACh release during development (Santafé et al., 2001, 2003, 2007b, 2009a) and in the adult (Santafé et al., 2005, 2006, 2007b). During NMJ synaptogenesis, the functional significance of the subtypes is different. M_2 receptors promote release in all nerve endings independently of their ACh release level or maturation state whereas an M_1- and M_4-mediated reduction in release is observed in the weakest endings on polyinnervated dual junctions (Santafé et al., 2001, 2002, 2003, 2004, 2007b, 2009a). Similarly, ARs are present in the motor terminals of the newborn and adult NMJs (Garcia et al., 2013, 2014). In the adult, the extent to which inhibitory A_1 and excitatory A_{2A} modulate the evoked release of ACh (Correia-de-Sá et al., 1991) seems to depend on the extracellular concentration of adenosine. During development, low concentrations of ADO may activate both A_1 and A_{2A} and have a facilitatory action on ACh release (Pousinha et al., 2010). Therefore, mAChRs and ARs (the autoreceptors of the transmitter ACh and the cotransmitter adenosine – derived from ATP hydrolysis – released by the active nerve endings themselves) are good candidates to be involved in postnatal axonal competition and synapse elimination. The involvement of these receptors may allow direct competitive interaction between nerve endings through a differential activity-dependent ACh and ADO release. So, the more active endings may directly punish the less active endings or reward themselves if the suitable mAChR and AR subtypes are present in the competing axons.

2.3.1. COOPERATION BETWEEN mAChRs AND ARs AT P7

At P7, about half of the axons are lost from the multiinnervated newborn NMJ (Lanuza et al., 2001). The selective inhibitors of the presynaptic mAChRs (M_1 and M_4 subtypes) and ARs (A_1 and A_{2A}) accelerated axonal elimination when applied on the LAL muscle surface between P5-P6. This means that the receptors slowed transition to monoinnervation in the untreated NMJ and thus reduced axonal loss (promoted axonal stabilization) when acting individually at least between P5-P6 (red arrows in **figure 47**). Slowing axonal elimination means the temporal retention of some – probably the weakest – axons by increasing their competitive force. By blocking only one of these receptors axonal loss increases (the 3-to-1 transition accelerates). Thus, all four receptors are necessary (though with some difference in potency) and if only one is blocked, axon loss will accelerate. At least partly for the purpose of synapse elimination, the four

operators may operate through the same intracellular mechanism. However, the sum of the individual effects of these receptors does not increase axonal retention (simultaneously blocking two receptors does not accelerate elimination more than blocking just one of them). Interestingly, the effect of blocking M_4 together with M_1 or A_1 is no different from the effect of blocking M_4 by itself but is significantly different from the individual effects of M_1 and A_1. This may mean that M_4 receptors are more prevalent than M_1 and A_1 receptors, which suggests that they may cooperate. Similarly, when AR inhibitors act together, the result is no different from when DPCPX acts by itself. However, it is significantly different from the individual effect of SCH, which suggests that the A_{2A} receptor needs the cooperation of the A_1 pathway for its potent effect. These cooperative relations between receptors are represented with blue links in **figure 47**.

Figure 47 shows an overall representation of the data. The thickness of the red arrows shows the relative individual ability of the mAChRs and ARs to delay axonal loss (the thicker they are, the greater their effect; the inhibitor used is noted in brackets under the receptor name). The links between the corresponding intracellular pathways may determine the delay in axon loss. It seems that all the receptors but M_2 (which does not directly affect axonal elimination because of the lack of effect of MET (black arrow in **figure 47**)) are involved in axonal competition.

As stated, our results show that all receptors use a common mechanism. These receptors are coupled to intracellular pathways that converge on a limited repertoire of effector kinases to phosphorylate protein targets and materialize structural and functional changes. M_1 and A_1 operate by respectively stimulating and inhibiting the PLCγ and PKC pathway whereas M_2-M_4 and A_{2A} operate by respectively inhibiting and stimulating the adenyl cyclase and PKA pathway (Caulfield MP, 1993; Felder, 1995; Caulfield and Birdsall, 1998; Nathanson, 2000). In all cases, however, common final changes such as intracellular calcium oscillations are observed (Santafé et al., 2006; Amaral and Pozzo-Miller, 2012). Interestingly, blocking all PKC isoforms, or the VDCCs (P/Q-, N- or L-type or Ca^{2+} influx) or mAChRs (M_1- and/or M_4-subtypes with PIR and MT3, respectively) results in an increase on the size of the synaptic potentials evoked by the weak axons in dual junctions whereas EPPs evoked by the strongest endings are reduced or unaffected (Santafé et al., 2003, 2004, 2007b, 2009a, 2009b; Tomàs et al., 2011). Thus, the difference in ACh release between competing endings is reduced by these muscarinic blockers, and this difference may change the competitive balance of the nerve terminals (at least in a muscarinic-dependent manner). However, as shown here, both PIR

and MT3 simultaneously accelerate axon loss at P7 and it is not clear how this acceleration is related to the changes in ACh release (increase in weak endings, decrease in strongest endings) and the presumed decrease in activity-related competition. The peculiar effect of the mAChR on the weakest endings may be related to the specific presence of the M_4 receptor subtype linked to P-, L- and N-type channels and an inhibitory PKC coupling to ACh release in these contacts (Santafé et al., 2009a).

The M_2 subtype is also present in the weak and strong axons in dual junctions (Santafé et al., 2003). This receptor links only with P- and N-channels and potentiates ACh release in both nerve endings but it has been shown that it does not directly affect axonal elimination at P7. However, **figure 47** shows a possible involvement of the M_2 receptors (because the M_2 inhibitor MET, in association with the other blockers, partially or totally occludes their individual effects). A simple explanation is that M_2 receptors modulate by a permissive action the other mAChRs and ARs (green arrows in **figure 47**; their thick indicate the relative modulatory potency in each case).

An alternative explanation is that the M_2 receptors at P7 have a more direct and active role in concordance with their active role at P9 (see later). The elimination-promoting effect of M_2 (dotted green arrow in **figure 47**), however, would be lower than the combined elimination-preventing cooperative effect of A_{2A}, M_1, M_4 and A_1. Therefore, M_2 by itself cannot accelerate elimination and blocking it with MET does not change axon loss. Blocking only one elimination-preventing receptor would release the axonal retention effect and allow M_2 to accelerate elimination. In this context, the observation that blocking A_{2A} or M_1 strongly accelerates axon loss suggests that these two receptors neutralize M_2. Because blocking M_2 with MET does not increase axonal retention by itself, the A_{2A}, M_1, M_4 and A_1 ensemble seems to operate at maximum capacity.

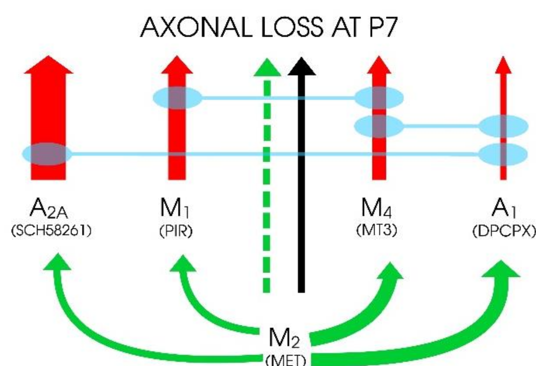


Figure 47. Diagram showing an overall representation of the cooperation between mAChRs and ARs at P7. Green and red arrows with different thickness indicate the relative individual potency of these receptors to accelerate or delay axonal elimination. The black arrow indicates that M_2 receptor does not affect the axonal loss. A blue bond indicates the association between these receptors. Dotted green arrow indicates that M_2 seems to accelerate the axonal loss process.

2.3.2. COOPERATION BETWEEN mAChRs AND ARs AT P9

The continued action of these receptors (including the M_2 mAChR subtype but not M_4) between P5-P9 finally promotes axonal loss and accelerates the monoinnervation of the NMJ (green arrows in **figure 48**). This means that the receptors accelerate the transition to monoinnervation and thus promote the unstabilization of some axons. All four receptors (M_2, M_1, A_1 and A_{2A}) are necessary (though with some variation in potency, which is indicated by the thickness of the green arrows in **figure 48**) and if only one is blocked, then axon loss is delayed. Also in this period, all receptors converge on a common mechanism.

The question at the end of the first postnatal week is why the blockers have such different effects on axon loss between P7 and P9. The receptors action may shift from delay axonal loss by favouring the competitive capacities in some nerve endings, to promote loss between P7-P9. In fact, one consequence of favouring initial competition around P7 would be an increase in axonal loss at P9. A developmental shift in the functional coupling of some molecules is not such a rare occurrence. Previous studies have shown changes in the role of the mAChRs themselves, the VDCCs (P, N and L) and the PKC during develoment depending on the maturation level of the NMJ. For instance, PKC couples to potentiate ACh release in the adult NMJ, in the strong ending of dual junctions and in the solitary

ending in the junctions monoinnervated during maturation. However, as discussed above, their coupling reduces release in the weakest axons in dual junctions and, therefore, any change in the functional expression of PKC may determine the regulation of axonal loss (Santafé et al., 2007a, 2009b).

The experiments clearly show real cooperation between M_1, A_1 and A_{2A} receptors. It has been associated one AR blocker (DPCPX or SCH-58261) with the M_1 blocker PIR. It has been found that both DPCPX and SCH-58261 add their delay on axonal loss to the delaying effect of PIR, two increases of roughly 58% and 36%, respectively. These results show an additive effect and indicate that at least these receptors cooperate (blue bonds in **figure 48**). However, the effect of the M_2 blocker MET is not modified by the presence of the M_1, A_1 or A_{2A} blockers, which indicates that the potent M_2 effect is independent of the other receptors (though, as stated, it seems to be partly modulated by M_4). When DPCPX and SCH act together (blue bond in the figure), the result is a percentage of monoinnervation that is no different from the control PBS, which indicates that both AR pathways are fully dependent on each other and need to cooperate if they are to regulate axon loss.

Whereas at P7, M_2 receptors seem to modulate the action of the other mAChR and AR, the M_4 receptor (which does not affect synaptic elimination at P9 by itself, black arrow in **figure 48**) can modulate other receptors at this time except M_1 (black dotted line in **figure 48**). Specifically, M_4 can cooperate positively with M_2 (dotted green arrow in this figure, because MT3 partly occludes the effect of MET). Also, the delaying effect on axonal loss of both DPCPX and SCH-58261 is potentiated (by about 30%) by MT3, which suggests a negative influence of M_4 on A_1 and A_{2A} receptor effects in normal conditions without any inhibitor present (red arrows in the figure).

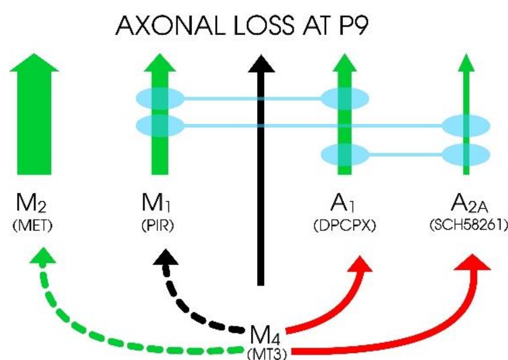


Figure 48. Diagram showing an overall representation of the cooperation between mAChRs and ARs at P9. Green and red arrows with different thickness indicate the relative individual potency of these receptors to accelerate or delay axonal elimination. The black arrow indicates that M_4 receptor does not affect the axonal loss. A blue bond indicates the association between these receptors. Dotted black arrow indicates that M_4 does not make any cooperation with M_1.

2.3.3. RELATION BETWEEN mAChRs AND ARs

In the adult, ARs and mAChRs heavily depend on each other to modulate ACh release by sharing the PKC and PKA pathways (Garcia et al., 2013; Santafé et al., 2015). The influx of external Ca^{2+} required for Ca^{2+}-triggered exocytosis and the fast mode of endocytosis seems to be promoted with the involvement of the presynaptic mAChRs (Santafé et al., 2006), and interaction with the ARs (Oliveira et al., 2009) but also with the BDNF/TrkB receptor pathway (Garcia et al., 2010e; Amaral and Pozzo-Miller, 2012). The mAChR and AR pathways (M_1 and A_1 receptors) share a link mediated by the set PLCγ-PIP_2-DAG-PKC, which modulates P/Q-type VDCC (Santafé et al., 2006; Amaral and Pozzo-Miller, 2012). The PLCγ-generated DAG also regulates the vesicle priming protein Munc13–1 and recruits ACh-containing vesicles for the immediately releasable pool (Bauer et al., 2007). M_2-M_4 and A_{2A} receptors share the PKA pathway. Previous findings show the influence of PKA activity on the coupling of PKC to ACh release and the coordinated involvement of PKC and PKA in the intracellular cascades that modulate neuromuscular transmission (Santafé et al., 2008). The action of the two kinases may be in the same molecules or in different molecules in the release machinery. A protein can be phosphorylated by at least two protein kinases, stimulated by different second-messenger systems, which exhibit both overlapping

and unique specificities for the phosphorylation of multiple sites in the molecule (Chambers et al., 1994). The complementary function of these receptors and kinases in the adult reinforces the suggestion that they may have a complementary function in developmental synaptic elimination. It has been shown here that postnatal axonal stabilisation or loss is a regulated multireceptor mechanism involving the cooperation of muscarinic (M_1, M_2 and M_4) and adenosine autoreceptor (A_1 and A_{2A}) subtypes in the motor nerve endings. New experiments can be performed to evaluate the downstream mechanism that couples receptors and kinases to the molecular targets responsible of axonal destabilization and retraction.

CHAPTER 3. Presynaptic muscarinic acetylcholine receptors and TrkB receptor cooperate in the elimination of redundant motor nerve terminals during development.

This chapter corresponds to the published article (see Appendix III):

Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Marta Tomàs, Maria A. Lanuza, Victor Cilleros, Josep Tomàs. (2017). Presynaptic muscarinic acetylcholine receptors and TrkB receptor cooperate in the elimination of redundant motor nerve terminals during development. Frontiers in Aging Neuroscience 9:24. DOI:10.3389/fnmol.2017.00132

3.1. BRIEF INTRODUCTION AND SCOPE

Chapter 1 has been shown how individual mAChR subtypes (M_1, M_2 and M_4), AR subtypes (A_1 and A_{2A}) and TrkB receptors are involved in the control of synapse elimination in the mouse NMJ. Moreover, in chapter 2, it has been investigated the additive or occlusive effects of the inhibitors of mAChRs and ARs, and thus the existence of real cooperation between them in synapse elimination at the NMJ. For instance, real cooperation between M_1, A_1 and A_{2A} receptors promotes axonal loss at P9, whereas the potent axonal loss-promoting effect of M_2 is largely independent of the other receptors. But, which is the relation between mAChR and TrkB receptor?

In this study, it has investigate whether the mAChR subtypes and the TrkB receptor also work together and whether the respective pathway inhibitors have any additive or occlusive effects and, therefore, whether there is any real cooperation between them in synapse elimination at the NMJ.

3.2. RESULTS

3.2.1. COOPERATION BETWEEN mAChRs AND TrkB RECEPTOR AT P9

In chapter 1 and 2, it has been observed that the mAChR subtype-selective inhibitors and TrkB blocker play a role in the synaptic competition process. But, it could be cooperation with these receptors? In chapter 1, it has been shown that pirenzepine (PIR, M_1 blocker) and methoctramine (MET, M_2 blocker) and the

RESULTS

TrkB pathway blocker used (a TrkB-Fc chimera), when applied one time each day between P5-P8 on the LAL muscle surface, results in a notable delay of the transition to monoinnervation on the NMJ observed at P9. However, the M_4 subtype blocker muscarinic toxin-3 (MT3) shows any effect on axonal loss at this time. However, the absolute potency of these blockers in modulating synapse loss cannot directly compared because the difference in the blocking efficacy of the respective selective inhibitors. Therefore, in normal conditions without inhibitors, the two receptor sets (mAChR and TrkB) will contribute to promoting the conditions of axonal disconnection at the beginning of the second postnatal week (see also in chaper 1).

Experiments were performed to investigate the effect on axonal loss of simultaneous incubation with two inhibitors (two antagonists of two different receptors) to reveal the possible occlusive or additive crosstalk effects between the corresponding pathways. **Figures 49** and **figure 50** show the effect of the drugs association applied between P5 and P8 and observed at P9. The **figure 49** shows the percentage of NMJs (the percentage of singly-, doubly- and triply -or more- innervated endplates) after the simultaneous inhibition of two receptors that, individually, clearly modulate axon loss (all except M_4). The associations of the M_4 blocker MT3 (that does not affect axonal elimination by itself) with the other substances has been represented in **figure 50** to make more understandable the data which reveal a complementary role for the M_4 subtype.

Figure 49 shows that the association of the mAChR blockers PIR and MET (**figure 49b**) does not show any additive effect or mutual occlusion in relation with axonal loss ($p>0.05$). However, the association of the M_1 and TrkB pathways inhibitors (PIR plus TrkB-Fc) results in a clear addition of their respective delaying effects on axonal loss (**figure 49c**). The percentage of the monoinnervated NMJ after the simultaneous exposition to both inhibitors is significatively less (25% of single junctions) than after exposition to PIR only (39%; $p<0.005$) or TrkB-Fc only (43%; $p<0.005$). Interestingly however, the individual effect of the TrkB-Fc, does not add to the effect of MET being the result of this dual drug incubation not different of the MET effect (**figure 49d**). The delaying effect of MET on axon loss is the most potent observed in the present experiments and is produced independently of the state of TrkB.

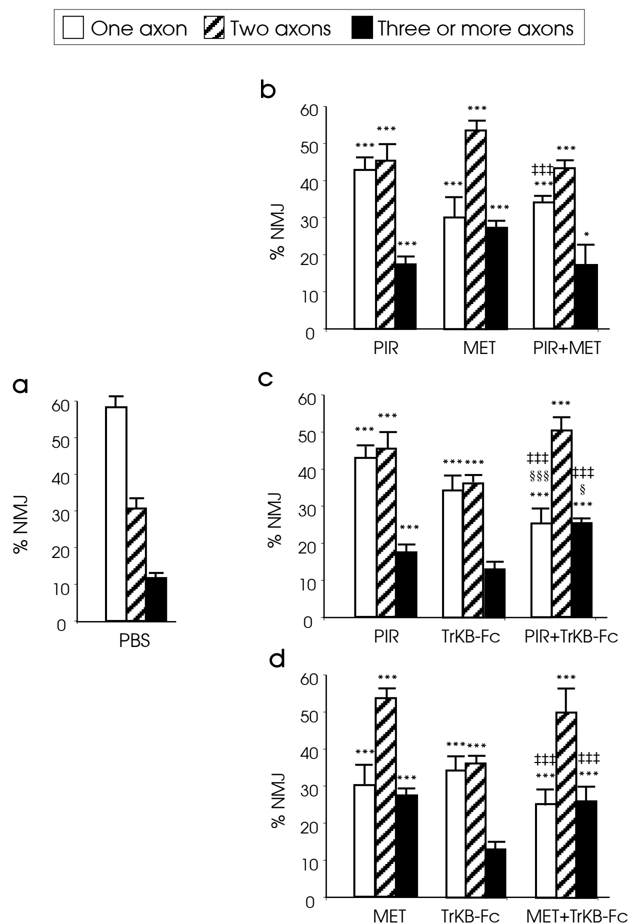


Figure 49. Cooperation between mAChRs and TrkB receptor at P9. The graphic shows the percentage of singly-, doubly- and triply- (or more) innervated NMJs after simultaneous inhibition of two receptors that individually affect axon loss (all the inhibitors but MT3). The symbols indicate: * $p<0.05$, ** $p<0.01$, *** $p<0.005$ when the correspondent antagonist or combinations of two substances is compared with control PBS. § $p<0.05$, §§ $p<0.01$, §§§ $p<0.005$ when the combination of two substances is compared with the first substance. ‡ $p<0.05$, ‡‡ $p<0.01$, ‡‡‡ $p<0.005$ when the combination of two substances is compared with the second.

M_4 blocker MT3 by itself does no produce any effect on axonal loss at P9. **Figure 50** shows that the simultaneous application of MT3 with the other blockers reveals some regulatory or complementary role of M_4 on the other receptors. The presence of MT3 does not change the effect of PIR (**figure 50b**) though some occlusion of the potent effect of MET (**figure 50c**) is observed which, however,

continues delaying significatively axon loss (the three-to-one transition). Interestingly, the presence of MT3 potentiates the delaying effect of TrkB-Fc (**figure 50d**) on axonal loss indicating the cooperation of the respective receptor path ways (M_4 and TrkB). MT3 with the presence of TrkB-Fc decrease the number of monoinnervated junctions (p<0.005) and increase the number of juctions innervated by three or more axons (p<0.005).

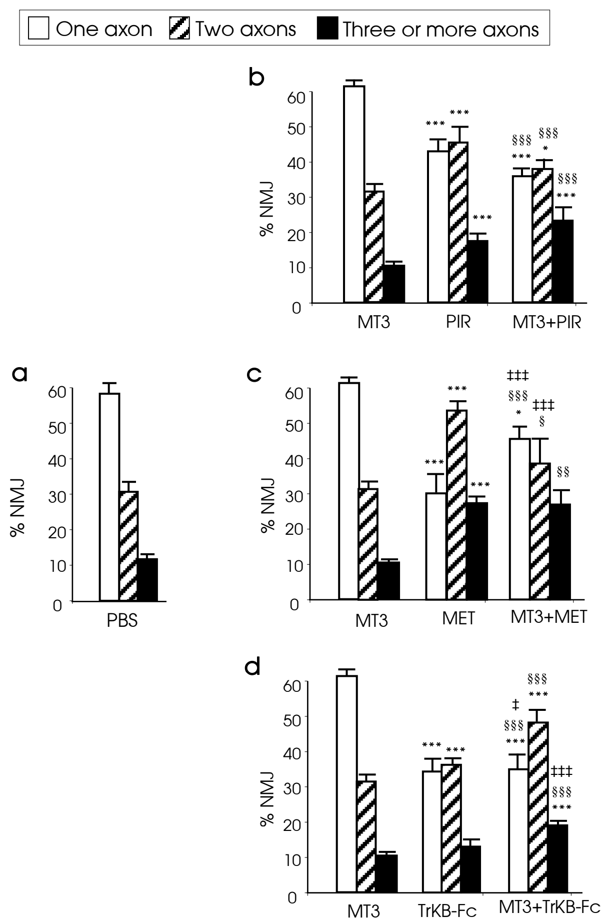


Figure 50. Cooperation between mAChRs and TrkB receptor at P9 (only MT3 with other substances). The graphic shows the percentage of singly-, doubly- and triply- (or more) innervated NMJs after simultaneous inhibition of two receptors that individually affect axon loss. It has been represented only associations of MT3 with the other substances. The symbols indicate: * p<0.05, ** p<0.01, *** p<0.005 when the correspondent antagonist or combinations of two substances is compared with control PBS. § p<0.05, §§ p<0.01, §§§ p<0.005 when the combination of two substances is compared with the first substance. ‡ p<0.05, ‡‡ p<0.01, ‡‡‡ p<0.005 when the combination of two substances is compared with the second.

3.3. DISCUSSION

The present experiments show evidence of the cooperation between the presynaptic M_1, M_2 and M_4 mAChR subtypes and the TrkB signaling to modulate the conditions of the developmental axonal competition and loss. In a previous study, it has been found that these receptors (as well as presynaptic adenosine receptors –AR-, A_1 and A_{2A} subtypes) separately contribute to accelerate synapse elimination around P9 in the mouse NMJ. It was thought that the muscarinic autoreceptors of the transmitter ACh may allow direct competitive interaction between nerve endings through a differential activity-dependent ACh release. The more active axons may directly punish the less active ones or reward themselves (Santafé et al., 2009a). However, an axon that is eliminated at one NMJ may be successful at another (Tomàs et al., 2011), which suggests that other receptors and local postsynaptic- (and glial cell) derived factors are involved. The involvement of the TrkB signaling described may allow a postsynaptic-derived trophic substance such as BDNF or NT4 to make a contribution (Yoshii and Constantine-Paton, 2010).

Interestingly, it has been observed that both the presynaptic-derived signal (ACh acting on axonal M_1 and M_2 mAChRs) and the TrkB-mediated signal (which may be originated by a postsynaptic-derived neurotrophin) have the same effect: namely, the acceleration of supernumerary nerve ending elimination. It seems that the outstanding regulatory resources in the NMJ synaptogenesis are committed to achieving monoinnervation. These presynaptic receptors converge in a common intracellular mechanism and a limited repertoire of effector kinases to phosphorylate protein targets and bring about structural and functional changes leading to axon loss. It is well known that in most cells M_1 and TrkB operate by stimulating the PLCγ and therefore the PKC pathway along with the IP_3 pathway, whereas M_2-M_4 inhibit the AC and PKA pathway (Caulfield MP, 1993; Felder, 1995; Caulfield and Birdsall, 1998; Nathanson, 2000). In all cases, however, common final changes such as intracellular calcium oscillations can occur (Santafé et al., 2006; Amaral and Pozzo-Miller, 2012). Both PKA and PKC activity changes have been shown to affect pre- and postsynaptic maturation (Lanuza et al., 2001, 2002). Our present data can be related with the intracellular coupling of the receptors to these serine kinases. Though the blocking efficacy of the selective inhibitors of the muscarinic receptors is not assessed here, M_2 increases the axonal loss rate most with a slight involvement of the M_4 receptor but independently of the M_1 and TrkB receptors. This suggests that downregulation of PKA activity through

the couple M_2-M_4 is a key factor in synapse elimination. Concurrently, M_1 and TrkB also contribute separately to axonal loss, but their combined action has a potent summed effect similar to the effect of the M_2 receptor. This suggest that activation of the PLCγ-PKC pathway through the couple M_1-TrkB may be the other key factor in this process. Thus, a displacement of the PKA/PKC activity ratio to lower values (inhibition of PKA and/or stimulation of PKC) in some nerve endings may have a leading role in synapse elimination. In this context, blockade of PKC in the newborn LAL muscle produces an initial blockade of synapse elimination and a subsequent delay (Lanuza et al., 2002).

In fact, these changes in the kinase activity leading to synapse elimination must occur at least (but not only) in the weakest axons during the competitive interactions. The neurotransmitter release capacity is an important factor in the competing capacity of the various nerve terminals in a NMJ. During development, in the polyinnervated NMJ several nerve endings with different levels of maturation and ACh release capacity get together and compete. The coupling to neurotransmitter release of the considered receptors and kinases is not the same in each of these various endings themselves and in the mature synapses in the adult (Tomàs et al., 2014). So, how does the specific coupling to ACh release of receptors and kinases in the weak and strong axons in competition contribute to axonal loss? As far as serine kinases are concerned, in the adult motor nerve endings both PKA and PKC potentiate ACh release when coupled to neurotransmission (Santafé et al., 2009b). Similarly, the same potentiation is observed in most neuromuscular synapses during development as, for instance, in those formed by the strongest axons (those that evoke the large end-plate potential, EPP) in the polyinnervated junctions (Santafé et al., 2006). However, in the weakest endings the inhibition of PKC increases the evoked EPP size indicating that, in normal conditions without any inhibition, this kinase tonically couples to ACh release reduction in these low releasing synapses. Therefore, an M_1-TrkB-mediated increase in PKC activity in the weakest endings would debilitate further their ACh release capacity and competitive force and facilitate their elimination. In addition, an M_2-mediated PKA downregulation in all nerve endings in competition may differentially affect their ACh release and contribute to elimination. Thus, at this point, there is a significant agreement between the known involvement of these molecules in neurotransmission and axon loss.

However, when considering the real postnatal coupling to ACh release of the mAChRs and the TrkB receptor in the different nerve endings (the strongest and the weakest) on developing synapses (Santafé et al., 2004, 2009a), additional interpretative keys are need. In the mature NMJ, M_1 and M_2 subtypes modulate evoked transmitter release by positive and negative feedbacks, respectively (Santafé et al., 2003, 2006). However, during NMJ synaptogenesis, the functional significance of the subtypes is different from in the adult. M_2 receptors promote release in all nerve endings independently of their ACh release level or maturation state whereas an M_1- and M_4-mediated reduction in release is observed in the weakest endings on dual junctions (Santafé et al., 2001, 2002, 2003, 2004, 2007b, 2009a). Similarly, the BDNF/TrkB pathway contributes to potentiate ACh release in different neuromuscular adult models but the potentiation is not observed in the weakest nerve endings during development and even some ACh release inhibition was observed in the strongest endings (Garcia et al., 2010d). Therefore, interpreting the links and molecular relations between transmitter release and elimination of nerve terminals seems more complex than it seemed at first. The involvement of other signaling such as AR can contribute to this complexity (Todd and Robitaille, 2006).

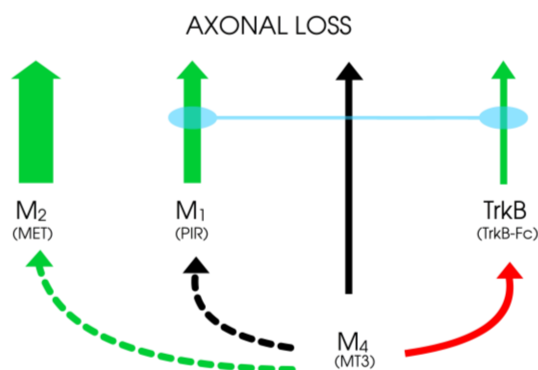
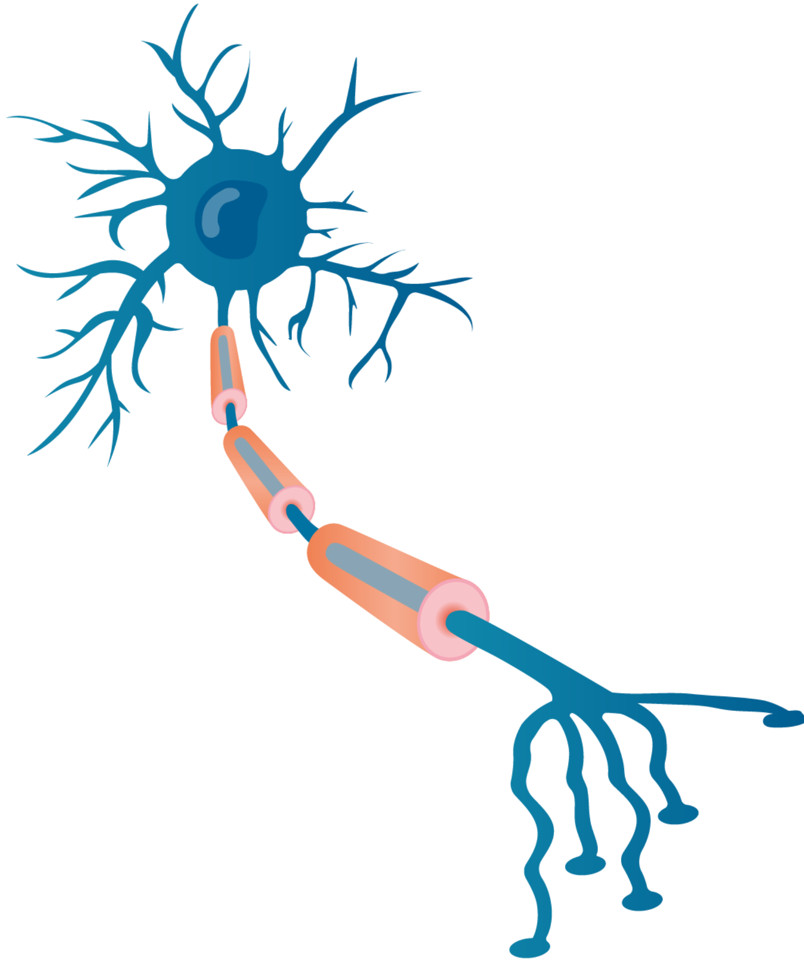


Figure 51. Diagram showing an overall representation of the cooperation between mAChRs and TrkB receptor at P9. Green arrows with different thickness indicate the relative individual potency of these receptors to accelerate axonal elimination. The black arrow indicates that M_4 receptor does not affect the axonal loss. A blue bond indicates the association between these receptors. Dotted black arrow indicates that M_4 does not make any cooperation with M_1.

However, some conclusions can be drawed taken in consideration all the aforesaid data. First, it seems outstanding that, contrary to what happens in the adult, M_1 (and M_4) and PKC activity reduce ACh release in the weakest endings in coincidence with the promotion of axonal loss. In fact, blocking mAChRs (M_1- and/or M_4-subtypes) or PKC or VDCCs (P/Q-, N- or L-type or Ca^{2+} influx) can lead to similar percentage increases in the size of the synaptic potentials evoked by weak axons (Santafé et al., 2003, 2004, 2007b, 2009a, 2009b; Tomàs et al., 2011). Therefore, the M_1-PKC pathway may debilitate the ACh release capacity and competitive force of these synaptic contacts and facilitate their elimination. The final target molecules involved may be the VDCC, specially the L-type which is exclusively coupled to ACh release in these endings (Santafé et al., 2001) and may contribute to carry high calcium near molecular mechanism relevant to axon loss. Second, the BDNF/TrkB signaling contributes to accelerate axon loss similarly to M_1 signaling. However, its involvement in the modulation of ACh release in the nerve endings that are in competition is less clear because does not affect release in the weak axons. Because PKC effectively reduces release in these endings, the TrkB pathway may operate through the IP_3 pathway to increase intracellular calcium and modulate the loss of axons. Third, M_2 has a strongest effect promoting axonal elimination. However, contrary to the adult, this muscarinic subtype promotes ACh release in all endings that are playing in competition, including the weakest endings and the solitary ending that finally wins the competition. Therefore, there is a shift of the M_2 coupling during development but how this affect their relation with PKA and how this relates with axonal loss is not known.



V. GENERAL DISCUSSION

In this thesis, it has been demonstrated by quantitative morphological studies the role of presynaptics mAChRs, ARs and TrkB receptor in the axonal loss at the neuromuscular junction of neonatal rodents. It is known that several signaling molecules and presynaptic receptors play a role in the axonal competition, which means that the various nerve endings influence one another (Santafé et al., 2009a; Garcia et al., 2011). This process is closely related to ACh release mechanism, which can be studied by electrophysiology. At the end of the first postnatal week, these receptors modulate transmitter release in the various nerve terminals on polyinnervated NMJ and contribute to axonal competition and synapse elimination. Is for this reason that in the following discussion has been deemed appropriate to link the morphological analysis with the functional studies on the neuromuscular junction. The following discussion is divided in two main parts. The first one concerns how individually presynaptic mAChRs, ARs and TrkB receptors are involved in ACh release and in the synaptic elimination process. The second part relates to cooperation of these receptors in the process of axonal loss, which is strongly associated with their signaling pathways. In addition, it has been proposed a molecular background for developmental axonal competition.

mAChRs M_1-, M_2- and M_4-subtypes in axonal loss

M_1, M_2 and M_4 mAChR subtypes are present in the NMJ (Garcia et al., 2005; Wright et al., 2009), regulate ACh release and play a role in the synapse elimination process during the development (Santafé et al., 2003, 2004, 2009a). In concrete, M_1 and M_4 subtypes are involved in a mechanism that delays axonal elimination at P7 whereas M_2 subtype has no effect on it (Axonal loss, **figure 52**). Nevertheless, the action of all muscarinic receptors as a whole indicates that the muscarinic mechanism increases the rate of axonal loss in dual junctions and, therefore, the final transition to the monoinnervation. It seems that NMJs with different maturation levels have different sensitivities to muscarinic regulation. An example of this is that M_1 and M_2 antagonists delay the transition two-to-one when applied between P5-P6 but, when applied at P9, they accelerate axonal elimination. The muscarinic mechanism appears to operate at close to maximum capacity and, therefore, may not be able to increase its efficacy beyond P7 with agonists like OXO. The levels of these receptors or differences in turnover rate could contribute to the different effects observed at P7 and P9. For instance, differences in the internalization and the turnover of the mAChR family members have been described (Reiner and Nathanson, 2012; Ockenga and Tikkanen, 2015) and

endocytosis may favor the coupling of the receptors to different signal transduction cascades (Pierce et al., 2001). The elimination process comes to its normal range at the end of the second postnatal week (P15). This suggests that other signaling mechanisms help to resolve the correct synaptic connectivity (Yang et al., 2009; Je et al., 2012, 2013).

In summary, a tonic muscarinic mechanism initially delays axonal elimination (a selective M_1-M_4 effect). However, the overall mAChR effect may accelerate the last phase of axonal disconnection, the two-to-one transition. Thereafter the muscarinic effect at around P9 clearly promotes elimination of all supernumerary nerve terminals (an M_1-M_2 effect).

Role of mAChRs in ACh release and recovery of silent synapses

The role of ACh release and recovery of silent synapses have been studied by the Histology and Neurobiology Unit through intracellular electrophysiological experiments. The procedure involved in detecting the function of polyinnervated synapses is complex. Is for this purpose that a short explanation of how they are studied is needed. It has been extensively described the characterization of the funcional capacity of the various motor axons that are in competition at the polyinnervated NMJ (Santafé et al., 2003, 2004, 2009a; Tomàs et al., 2011) using electrophysiological experiments in *ex vivo* LAL muscles from P6-P7 mice or rat. Briefly, after preventing contractions, the nerve is stimulated with an increasing intensity from zero until an EPP is observed. If the size and latency of the EPP remains constant as the stimulus is increased, it can be concluded that the endplate is monoinnervated (M endings). In endplates with polyneuronal innervation, increasing the stimulus amplitude causes one or more axons to be recruited, which produces a stepwise increment in the EPP (Redfern, 1970). Specifically, with dually innervated fibers (the most affordable polyinnervation condition), a second EPP can appear after the first one when the intensity of the electrical stimulus is increased. This compound EPP is built by recruiting two axons. The EPP amplitude of the second axon response can be calculated by subtracting the first EPP amplitude from the compound EPP. Usually, these EPPs have different amplitudes because their size is not related to the threshold of the axon that produces it. It has been referred to the axon terminals that produce these synaptic potentials as the weak (W, smallest EPP) and strong (S, largest EPP) nerve endings. In addition, it has been observed (Santafé et al., 2009a; Tomàs et al., 2011) that some nerve terminals go silent (do not evoke EPP on stimulation) before they completely retract and

before the end of the functional elimination period, but retain certain capabilities for evoked release that can be pharmacologically recovered (R, recovered endings). Also, intracellular recordings of the evoked synaptic potentials have been used to observe the number of functional inputs for a large number of NMJs. The mean value was defined as the polyinnervation index of the muscle studied (PI in control P6-P7 rodent muscles was 1.63 ± 0.14 with a 47.92% ± 2.08 of monoinnervated junctions; Lanuza et al., 2001; Santafé et al., 2001). Finally, the PI was used to study how several key molecules involved in ACh release can be related to synapse elimination by blocking or activating them. A rapid increase in the PI can indicate the recruitment of some silent nerve endings that transitorily recover transmission (R endings).

Using this procedure it was observed that, in the adult NMJ (A, **figure 52**), M_1 and M_2 receptors modulate evoked transmitter release by a positive and negative feedback, respectively (Slutsky et al., 1999; Minic et al., 2002; Santafé et al., 2003, 2006). Thus, in the mature NMJ the whole outcome of the mAChR signaling seems to save the synapse function by decreasing the extent of evoked release in basal conditions. However, during development, the involvement of mAChR in ACh release is different. At P6-P7 roughly half NMJ have become monoinnervated because one nerve terminal wins the axonal competition process (Lanuza et al., 2002; Santafé et al., 2002). In these axons (M, **figure 52**), all selective M_1 and M_2 blockers tested reduce release and noteworthy the same occurs in the strongest endings in dual junctions still in competition (S, **figure 52**). This suggests that a positive value of the winner axons can be that all functional mAChR are committed to enhance ACh release (in M and S contacts, the M_3 and M_4 blockers, do not affect release). Using this autocrine mechanism, the strongest endings may reinforce themselves. However, in the weakest nerve terminal in dual junctions (W, **figure 52**), only M_2 blockers reduce release whereas M_1 and M_4 blockers can lead to similar percentage increases in the size of the synaptic potentials evoked by weak axons (Santafé et al., 2003, 2004, 2007b, 2009a, 2009b; Tomàs et al., 2011).

In electrophysiological experiments, an increase in PI indicates a quickly recruitment of some silent synaptic contacts that transitorily recover transmission (R in **figure 52**). In P6-P7 muscles, it has been observed that stimulation of all mAChR with OXO does not change the mean PI but the percentage of functional monoinnervated NMJ increases dramatically (Tomàs et al., 2011). On the other hand, the blockade of all mAChR with AT increases the NMJ with 3-4 inputs and increases PI. This effect can be almost exactly reproduced by selectively blocking

M_2 with MET but not with MT7 (M_1 blocker) or MT3 (M_4 blocker). Thus, M_2 has a role on the recovery of silent synapses and would be involved in promoting the last step of the functional axonal disconnection. Whereas M_2 could stimulate release in M, S and W axons, this receptor subtype seem to reduce it in silent endings because the block of M_2 (MET) increases release in these endings just to be functionally recovered.

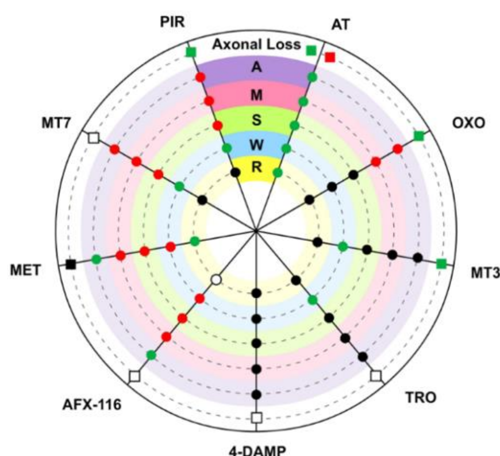


Figure 52. Effect of several subtype-selective and unselective muscarinic substances. The M_2 receptor is selectively blocked with methoctramine (MET) or AFX-116. The M_1 receptor is selectively blocked with pirenzepine (PIR) or muscarinic toxin 7 (MT7). The M_3 subtype is blocked with 4-DAMP and the M_4 subtype is blocked with tropicamide (TRO) or muscarinic toxin 3 (MT3). EPP size is represented with circles; increase –green–, decrease –red– and no change –black–. In developing (P7) single axons on monoinnervated junctions –M–, the strong –S– and weak –W– synaptic contacts on dual junctions and in adult (P30) nerve endings –A–. Silent synaptic contacts –R– can be observed in some NMJs of treated muscles after recovering ACh release. Here, R shows the effect of some substances in the polyinnervation index (PI, the mean number of axons per synapse) of these treated muscles. Axonal loss rate (represented with squares) is quantified by direct axonal counts in confocal LAL preparations from B6.Cg-Tg (Thy1-YFP)16 Jrs/J mice. Delayed axon loss, in red squares, accelerated loss in green and no change in black squares.

Relation between mAChR-mediated changes in axonal loss and ACh release

A relation between ACh release modulation and the delay in axon elimination may be hypothetically expected by mAChRs. At P7, the release capacity of the weakest endings is increased by the inhibitors PIR and MT3, whereas ACh release from the strong ending is reduced (by PIR) or unaffected (by MT3)

(Santafé et al., 2009a). Thus, the difference in ACh release between competing endings (S and W) is reduced, which may change the competitive balance of the nerve terminals. It has not been known exactly what the release capacity of the weak and strong endings is in the LAL muscle at P9. However, both PIR and MT3 accelerate axon loss at P7 and how this is related to the presumed lesser activity-related competition is not clear. A plausible interpretation is that in this developmental stage (P7), mAChR-mediated competition is fully operative in the NMJ of untreated muscles, and some axons, engaged in competition, have not been fully lost. If competition is reduced or unbalanced by, for instance, blocking M_1 or M_4 the loss of these axons accelerates. Also, in dual junctions ACh release is reduced by the M_2 blocker MET in both the weak and strong endings suggesting that the axonal difference in release is the same but axons are not as strong or have less competitive force. In this case, as may be expected, MET does not affect axonal elimination at P7. Thus, the relation between the ACh release capacity of the endings in competition and the rate of axonal loss in multiinnervated junctions seems to be best observed at P9 when, judging by the effects of PIR and MET, the receptors M_1 and M_2 play a role in accelerating axonal loss. The functional effect on ACh release of these receptors may reinforce the strongest endings and be detrimental to the weak endings in dual junctions.

ARs A_1-, A_{2A}-subtypes in axonal loss

ARs are present in the motor terminals of the newborn and adult NMJs (Garcia et al., 2013, 2014). A_1 has been localized in the terminal teloglial Schwann cell and in the nerve terminal, whereas A_{2A} has been found in the postsynaptic muscle, the axon and the nerve terminal (Garcia et al., 2013). However, there is not any information about the effect of agonists or antagonists of these receptors in describing the funcional capacity of the various motor axons that are in competition at the polyinnervated NMJ. Nevertheless, it is known that through A_1 and A_{2A} receptor activation, adenosine modulates neuronal homeostasis and plays a key role in synapse plasticity (Dias et al., 2013). These receptors can collaborate with mAChR to reduce depression during repetitive activity (Correia-de-Sá et al., 1991; Garcia et al., 2013; Santafé et al., 2014). In 3–4-week-old rats, low extracellular concentrations of ADO may activate both A_1 and A_{2A} and have a facilitatory action on ACh release. Also, blockade of A_1 receptors prevents both excitatory and inhibitory effects, whereas blockade of A_{2A} receptors prevents only

the excitatory effects (Pousinha et al., 2010). In the Histology and Neurobiology Unit, it has been found that in the adult NMJ the non-selective block of adenosine receptors with 8-SPT and the non-selective physiological agonist adenosine does not change evoked ACh release (Garcia et al., 2013). It has also been investigated the selective involvement of the A_1 and A_{2A} subtypes. Neither the selective block of A_1 with DPCPX nor A_{2A} with SCH-58261 have results in a significant change in evoked ACh released (Garcia et al., 2013). These findings confirm the previous observation that in resting conditions ARs are not tonically or constitutively coupled to any immediate modulation of stimulus-induced neurotransmission. In addition, the imbalance per se of the A_1 and A_{2A} mechanisms using the selective blockers and stimulators does not reveal any involvement of the endogenous adenosine in resting conditions. Moreover, a collaborative action between different AR subtypes reduced synaptic depression at a moderate activity level (40 Hz). Moreover, at high activity levels (100 Hz), endogenous adenosine production in the synaptic cleft was sufficient to reduce depression through A_1 and A_{2A}. When the non-metabolizable 2-chloroadenosine (CADO) agonist was used, both the quantal content and depression were reduced and the protective effect of CADO on depression was mediated by A_1, whereas A_{2A} seemed to modulate A_1 (Santafé et al., 2015).

In this thesis, we found that mAChRs and ARs delay axonal loss at P7 (although mAChR accelerate the last phase of axonal disconnection) but accelerate it at P9. There is an additive effect between M_1 and A_1 or A_{2A} which is an indication of the cooperation between at least these receptors. At P7, specific antagonists of ARs (DPCPX and SCH-58261) accelerate axonal elimination on the NMJ. Moreover, ADO, an agonist of AR induced retardation of axonal elimination. Therefore, the physiological role in normal conditions of the AR seems to delay the axonal loss process. However, at P9, the purinergic mechanism also seems to tonically accelerate axonal elimination to the maximum rate because the AR inhibitors delay the process. Therefore, it seems that AR may behave biphasically in the critical period between 5-9 postnatal days. An initial delay in axonal loss at P7 (an A_1- and A_{2A}-mediated effect which can be reinforced by exogenously added ADO) is followed by an A_1- and A_{2A}-mediated tonic acceleration at P9.

TrkB receptor in axonal loss

BDNF and its receptors have been shown to be expressed in muscle and nerve tissues both during development and adulthood (Garcia et al., 2010f). In

order to study the effect of TrkB receptor in axonal loss, TrkB-Fc has been used to sequester endogenous BDNF/NT4 neurotrophins. In the morphological analysis, it has been observed a clear acceleration of the three-to-two rate well matched by an acceleration of the two-to-one rate at P7 (Axonal loss, **figure 53**). Therefore, the physiologic role in normal conditions of the BDNF/TrkB pathway at P7 seems to produce a retardation of the axonal loss process though endogenous BDNF does not affect the ACh release in the W endings as stated above. This result agrees with a proposed model in which proBDNF and mature BDNF (mBDNF) serve as potencial 'punishment' and 'reward' signals for inactive and active terminals, respectively *in vivo* (Je et al., 2012). Exogenous proBDNF promoted synapse elimination via activation of $p75^{NTR}$ receptors, whereas mBDNF infusion substantially delayed synapse elimination in the mouse LAL muscle (Je et al., 2013). Also, the block of the $p75^{NTR}$ receptors in the LAL muscle of the mouse results in a delay of axonal loss and even some regrowth of nerve terminals (Garcia et al., 2011). However, at P9, neurotrophin signaling seems to reverse their coupling to the axonal loss process because TrkB-Fc strongly retard elimination (resulting in more dual and less monoinnervated NMJ). This indicates that, in the normal situation, the role of BDNF/NT4 mediators shift at P9 to accelerate elimination, similarly to the muscarinic mechanism described above.

Thus, also in this case, it seems that the BDNF/TrkB pathway may play a biphasic role during the critical period of synapse loss. The progressive maturation of the NMJ at P9 may change the operation conditions of the BDNF/TrkB pathway to a more mature endogenous BDNF production and release-promoting effect in certain endings resulting in more efficient competition and axonal elimination.

Role of TrkB-BDNF in ACh release and recovery of silent synapses

As mAChRs, the role of TrkB receptor in ACh release has been studied in newborn and adult junctions. In the adult NMJ (A, **figure 53**) of rodents, exogenously added BDNF (or NT-4) increases evoked ACh release after 3 hours (Mantilla et al., 2004; Garcia et al., 2010d). This presynaptic effect is prevented by preincubation with TrkB-Fc chimera or by pharmacological block of TrkB signaling (k252-a or the antibody blocker 47/TrkB). However, low doses of BDNF rapidly induce (within minutes) a TrkB-dependent potentiation at developing neuromuscular junctions in *Xenopus laevis* in culture (Stoop and Poo, 1996; Poo and Boulanger, 1999; Poo, 2001). In P7 developing muscles *ex vivo* (M, **figure 53**),

exogenous BDNF (10 nM for 3 hours or 50 nM for 1 hour) potentiates release in all endings also with the involvement of TrkB (Garcia et al., 2010d).

Thus, exogenous BDNF acts on a section of the release mechanism that is operative and potentiates neurotransmission in all nerve endings that are in developmental competition (regardless of their particular maturation state). However, when analysing the effect of the endogenous BDNF during synaptic maturation, blocking TrkB (K-252a) or neutralizing endogenous BDNF (TrkB-Fc), does not change the quantal content of the W endings though, surprisingly, increases release in the S endings (Garcia et al., 2010d) (S and W, **figure 53**). Therefore, though the BDNF/TrkB pathway seems ready to be stimulated by exogenous BDNF to potentiate release in all nerve terminals during development, endogenous BDNF does not affect the weak ending at P7 but, at this developmental period, may contribute to reduce release in the strongest ending (Garcia et al., 2010d). The effect of endogenous BDNF in S endings may be related with the relative involvement of truncated and full-length TrkB receptors, $p75^{NTR}$ receptors, and proBDNF and mature BDNF in the postnatal multiinnervated synapses.

Blocking TrkB or preventing endogenous BDNF action by using TrkB-Fc does not change the mean PI. However, stimulation with exogenous BDNF (one hour in the bath) transitorily increases PI with an important reduction of monoinnervated junctions and an increase of the 2-3 functional input junctions (Tomàs et al., 2011; R, **figure 53**). This suggests the presence of a number of silent inputs in the boundary to be recovered (to produce an EPP) by BDNF application. In fact, BDNF transiently stabilizes silent synapses at developing mice NMJ (Kwon and Gurney, 1996; Garcia et al., 2010d). It may be hypothesised that, because the low activity in the weakest endings, the production of BDNF is scarce and does not work locally on them. However, exogenous BDNF may reach the weak endings close to elimination and induce some release recovery. As previously stated, the downregulation of M_2 (MET) produces the same effect that TrkB stimulation with BDNF, that is to say an increase in PI by the recovery of certain transmitter release capacity in silent endings. ACh from the strong and more active terminals may reach M_2 in the silent endings thus punishing them.

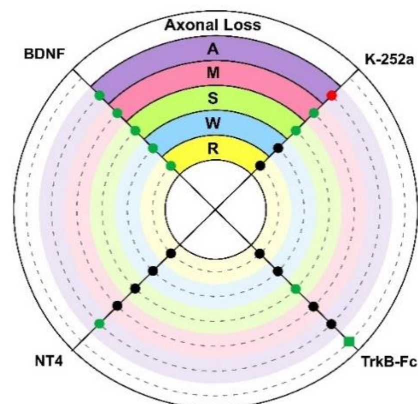


Figure 53. Effect of neurotrophins (BDNF and NT4) and related substances (TrkB-Fc chimera and K-252a). EPP size is represented with circles; increase –green–, decrease –red– and no change –black–. In developing (P7) single axons on monoinnervated junctions –M–, the strong –S– and weak –W– synaptic contacts on dual junctions and in adult (P30) nerve endings –A–. Silent synaptic contacts –R– can be observed in some NMJs of treated muscles after recovering ACh release. Here, R shows the effect of some substances in the polyinnervation index (PI, the mean number of axons per synapse) of these treated muscles. Axonal loss rate (represented with squares) is quantified by direct axonal counts in confocal LAL preparations from B6.Cg-Tg (Thy1-YFP)16 Jrs/J mice. Delayed axon, loss in red squares, accelerated loss in green and no change in black squares.

Relation between TrkB-mediated changes in axonal loss and ACh release

Electrophysiological recordings have determined that BDNF increases ACh release in both the weak and strong endings at P6-P7 (Garcia et al., 2010d). In addition, exogenous BDNF increases the percentage of functional polyinnervated junctions (Tomàs et al., 2011). Interestingly, exogenous BDNF infusion delays synapse elimination in the mouse LAL muscle (Je et al., 2013). The delaying effect of the TrkB pathway on axonal elimination at P7 described here may be related with the BDNF potentiation of the weakest endings about to be eliminated. However, blocking the TrkB receptor or neutralizing endogenous BDNF with the TrkB-Fc chimera at P7 does not affect the quantal content of the weak endings but increases the release in the strong ending, which suggests that endogenous BDNF, in this developmental period, may surprisingly reduce the release in the strongest ending (Garcia et al., 2010d). The delaying effect of the TrkB pathway on axonal elimination at P7 may be related to the BDNF-mediated lesser release and presumed lesser competitive force of the strong axon. The TrkB

pathway accelerates elimination at P9. The progressive maturation of the NMJ at P9 may change the operation conditions of the BDNF/TrkB pathway in the strongest endings resulting in more efficient competition and axonal elimination (Mantilla et al., 2004; Garcia et al., 2010d).

Relation between mAChR and AR signaling

It has been shown that postnatal axonal stabilization or loss is a regulated multireceptor mechanism involving the cooperation of muscarinic (M_1, M_2 and M_4) and adenosine autoreceptor (A_1 and A_{2A}) subtypes in the motor nerve endings. Once having seen the role of these three sets of receptors individually, we proceed to unveil the possible cooperation between these receptors, that could share signaling pathways to modulate the ACh release and, indeed, the axonal loss. In this thesis it has been demostrated that at P7, the effect of blocking M_4 together with M_1 or A_1 is not different from the effect of blocking M_4 by itself but is significantly different from the individual effects of M_1 and A_1. This may mean that M_4 receptors are more prevalent than M_1 and A_1 receptors, which suggests that they may cooperate. Similarly, A_{2A} receptor needs the cooperation of the A_1 pathway for its potent effect. Moreover, M_2 receptor modulates by a permissive action the other mAChR and AR. M_2 receptor induces an elimination-promoting effect which would be lower than the combined elimination-preventing cooperative effect of A_{2A}, M_1, M_4 and A_1. At P9 exists a cooperation between M_1, A_1 and A_{2A} receptors. However, the effect of the M_2 blocker is not modified by the presence of the M_1, A_1 or A_{2A} blockers, which indicates that the potent M_2 effect is independent of the other receptors. Both AR pathways are fully dependent on each other and need to cooperate if they are to regulate axon loss. M_4 can cooperate positively with M_2 and delay axonal loss when cooperates with A_1 and A_{2A}, which suggests a negative influence of M_4 on A_1 and A_{2A} receptors. However, new experiments should be performed to clarify the downstream mechanism that couples mAChRs and ARs with kinases to the molecular targets responsible of axonal competition.

mAChR and AR receptors are coupled to intracellular pathways that converge on a limited repertoire of effector kinases to phosphorylate protein targets and materialize structural and functional changes. These receptors signaling pathways share PKC and PKA pathways to modulate ACh release (Garcia et al., 2013; Santafé et al., 2015). Nowadays, there are not any data of specific studies which show the interaction between muscarinic and adenosine

receptors and the common signaling pathways during the period of synapse elimination. However, it is known that membrane receptors converge in a limited repertoire of effector kinases (mainly PKA and PKC) to phosphorylate protein targets and bring about structural and functional changes leading to axon loss. The nerve endings that lose the competitive process progressively weaken by diminishing the quantal content of the evoked ACh release in parallel with the progressive loss of nAChR from the postsynaptic muscle cell. In addition, serine kinases in the nerve terminals could be directly involved in modulating calcium-depending ACh release at the NMJ (Lanuza et al., 2014) and, specifically, reducing ACh release capacity of the weak axons in developing polyinnervated synapses (Santafé et al., 2003, 2006, 2007b, 2009a, 2009b; Tomàs et al., 2011). However, more study is needed to fully assess the effect of these receptors in the signaling pathways. Nevertheless, a displacement of the PKA/PKC activity ratio to lower values (inhibition of PKA and/or stimulation of PKC) in some nerve endings may have a leading role in promoting synapse elimination. Thus, mAChRs (Santafé et al., 2006) and ARs (Oliveira et al., 2009) promote the influx of external Ca^{2+} in order to trigger exocytosis. It has been demonstrated that in the adult skeletal NMJ, mAChR and AR pathways (M_1 and A_1 receptors) share a link mediated by the set PLCγ-PIP_2-DAG-PKC, which modulates P/Q-type VDCC (Santafé et al., 2006; Amaral and Pozzo-Miller, 2012). On the other hand, M_2-M_4 and A_{2A} receptors share the PKA pathway which modulates neuromuscular transmission (Santafé et al., 2009b). Thus, the result could be through at least two protein kinases, stimulated by different second-messenger systems, which exhibit both overlapping and unique specificities for the phosphorylation of multiple sites in their substrates (Chambers et al., 1994).

To sum up, we suggest that M_1 and A_1 receptors can operate by stimulating PLCγ and, therefore, the PKC pathway whereas A_{2A}, M_2 and M_4 along the PKA pathway.

Relation between mAChR and TrkB signaling

It can also be interesting to study the interaction between mAChR and TrkB receptor pathways because it has been already proven the role of these receptors individually in the axonal loss. M_2 increases the axonal loss rate the most with a slight involvement of the M_4 receptor but independently of the M_1 and TrkB receptors. This suggests that downregulation of PKA activity through the couple M_2-

M_4 is a key factor in synapse elimination. Concurrently, M_1 and TrkB also contribute separately to axonal loss and their combined action has a potent summed effect similar to the effect of the M_2 receptor. This suggests that activation of the PLCγ-PKC pathway through the couple M_1-TrkB may be the other key factor in this process. Thus, a displacement of the PKA/PKC activity ratio to lower values in some nerve endings may have a leading role in synapse elimination. Moreover, it has been observed that M_4 regulate negatively the TrkB receptor whereas M_4 does not change M_1 effect on axonal loss.

Previously, it has been demonstrated that the global outcome of the mAChR seems to protect the synapse function by decreasing the extent of evoked release (mainly an M_2 action) and reducing depression (Santafé et al., 2003). A main role of TrkB can be to maintain a low level of spontaneous quantal leak of ACh and potentiate evoked release (Garcia et al., 2010e). Thus, some functions in the adult can be balanced by the opposed action of different receptors. Changes in the operation of any of these pathways and their receptors affect the normal coupling of the other complementary molecules to transmitter release. Consecutive incubations with two substances (for instance a muscarinic blocker followed by a TrkB blocker) have been used as a pharmacological tool to investigate the possible occlusive or additive crosstalk effects between two receptors. A close dependence has been found between mAChR and TrkB receptor pathways because the normal function of the mAChR mechanism is a permissive prerequisite for the TrkB pathway to couple to ACh release and reciprocally (Garcia et al., 2010e; Santafé et al., 2014). mAChR and TrkB pathways share a link mediated by PLCγ-PIP_2-DAG-PKC, which leads to the modulation of P/Q-type VDCC (**figure 54**; Santafé et al., 2006; Amaral and Pozzo-Miller, 2012). Also, the PLCγ-generated DAG regulates the vesicle priming protein Munc13–1 and recruits ACh-containing vesicles for the immediately releasable pool (Bauer et al., 2007). Thus, the relations between these signaling pathways modulate VDCC and the ready releasable pool of synaptic vesicles, which are the instruments of neurotransmission (Takamori, 2012). The influx of external Ca^{2+} required for exocytosis seems to be promoted by the activation of presynaptic M_1 mAChR (Santafé et al., 2006) and interaction with the BDNF/TrkB receptor (Amaral and Pozzo-Miller, 2012). In the adult skeletal NMJ, the M_1 mAChR balances the M_2 mAChR, which functions as a PKA-mediated inhibitor of ACh release under physiological conditions (Santafé et al., 2006). This balance is modulated by adenosine coreleased with ACh at the NMJ (Oliveira et al., 2009; Garcia et al.,

2013; Santafé et al., 2015) and BDNF (Garcia et al., 2010e). However, when neuromuscular transmission is low (as during synaptic development) or defective, the balance between them shifts in favor of the M_1 mAChR, partly caused by an M_2 mAChR-mediated switch from PKA to PKC activation (Santafé et al., 2009b).

The complementary function of these receptors in the adult NMJ neurotransmission reinforces the indication of their coordinated involvement in developmental synaptic elimination. It has been suggested that this mechanism plays a central role in the elimination of redundant neonatal synapses. However, at P7, the coperation of M_1, M_4 and TrkB receptors delays axon loss at the same time that M_1 and M_4 reduce ACh release in the weakest axon terminals suggesting some independence between transmitter release and elimination. It has been interpreted that at this developmental point, the activity-dependent competitive interactions in most junctions are at its peak and this could result in delayed axon loss. The effect of these receptors in finally accelerating axon loss is more clearly manifested at P9.

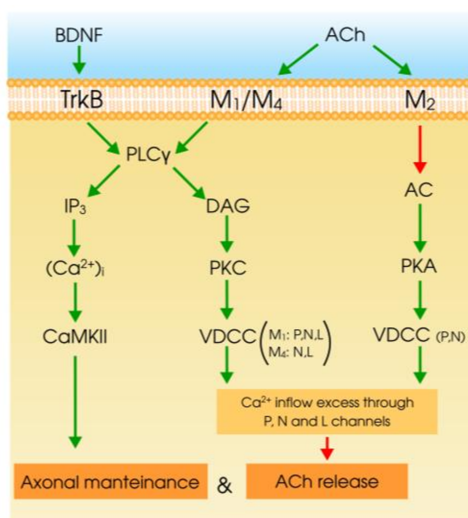
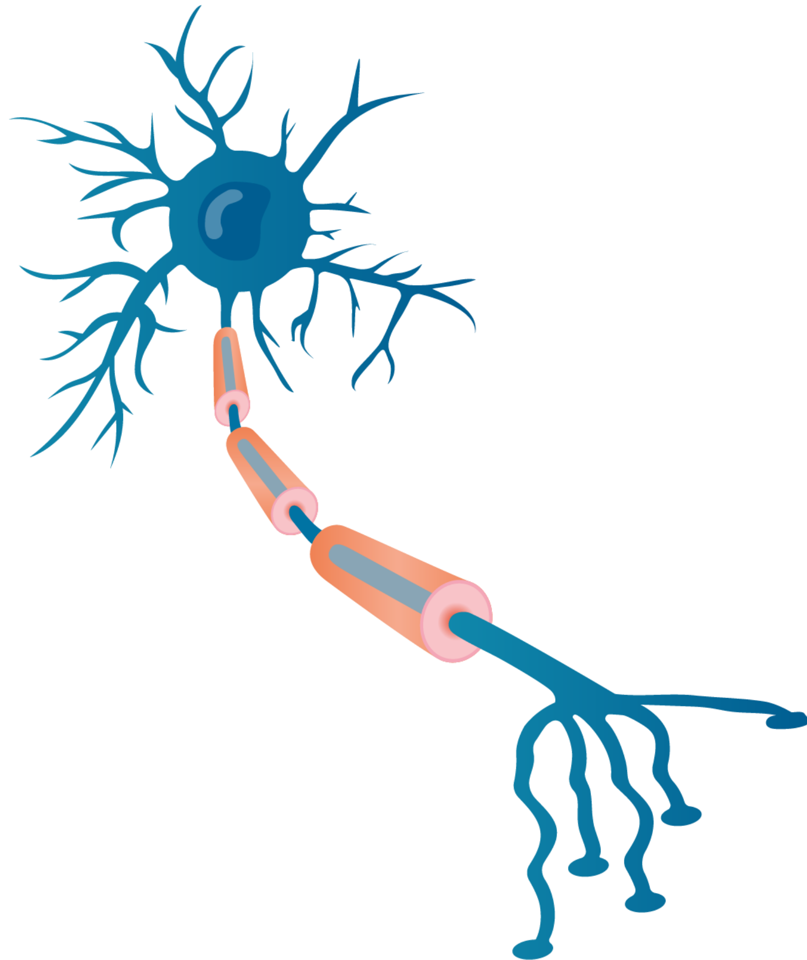


Figure 54. Plausible interpretation of the role of mAChRs and the BDNF/TrkB pathways in the process of elimination of the weakest endings around P7. M_1, M_4 and TrkB operate by stimulating the phospholipase C (PLCγ) and therefore the protein kinase C (PKC) pathway along with the inositol trisphosphate (IP_3) pathway, whereas M_2 inhibit the adenylyl cyclase (AC) and protein kinase A (PKA) pathway. Finally, these receptors caused changes in axonal maintenance and acetylcholine (ACh) release.



VI. CONCLUSIONS

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Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process
Laura Nadal Magriñà

OBJECTIVE 1. To know the effect of the individual mAChRs (M_1, M_2 and M_4), ARs (A_1 and A_{2A}) and TrkB signaling in the control of the synapse elimination during development. Identify how the modulation of these pathways modifies the number of motor nerve terminals per synapse at P7 and P9.

mAChRs, ARs and TrkB modulate the elimination process and promote axonal disconnection at the beginning of the second postnatal week.

- mAChRs, ARs and TrkB signaling are implicated in i) reducing the initial chance of elimination at P7 but ii) increasing thereafter axonal competition and elimination at P9.
    - M_1 and M_4 mAChRs subtypes are involved in a selective mechanism that delays axonal elimination at P7 whereas M_1 and M_2 subtypes cooperate to favour synapse elimination at P9.
    - ARs (A_1 and A_{2A}) and TrkB pathways at P7 produce a delay of the axonal loss process and accelerate it at P9.

OBJECTIVE 2. To know the effect of the individual mAChRs (M_1 and M_2), ARs and TrkB receptor signaling on the evolution of the number of motor nerve terminals per synapse at P15.

M_1, M_2, AR and TrkB inhibitors did not affect the synapse elimination process at the end of the second postnatal week (P15).

OBJECTIVE 3. To determine the involvement of mAChRs (M_1, M_2 and M_4), ARs (A_1 and A_{2A}) and TrkB receptor on maturation rate of nAChRs postsynaptic cluster at P7, P9 and P15.

The axonal elimination process is in some cases independent of the postsynaptic maturation of the nicotinic receptor cluster.

- M_1 and M_2 mAChRs delay the maturation of nAChRs at P7 while M_1, M_2 and M_4 at P9.
- M_1 and M_2 mAChRs produce a delay in the maturation of nAChRs at P15.
- A dual effect of the TrkB pathway (hasten at P7, delay at P9) and AR-mediated hastens maturation of nAChRs during the P7-P9 period.
- ARs and TrkB signaling pathways are necessary in the maturation of nAChRs during the second postnatal week.
    - ARs accelerate the postsynaptic maturation of nAChRs at P15 when they are blocked with non- selectitive antagonist (8STP).
    - TrkB accelerates the postsynaptic maturation of nAChRs at P15.

OBJECTIVE 4. To know the interaction between mAChRs (M_1, M_2 and M_4) and ARs (A_1 and A_{2A}) signaling in the modulation of the synapse elimination during development. To identify the number of motor nerve terminals per synapse at P7 and P9.

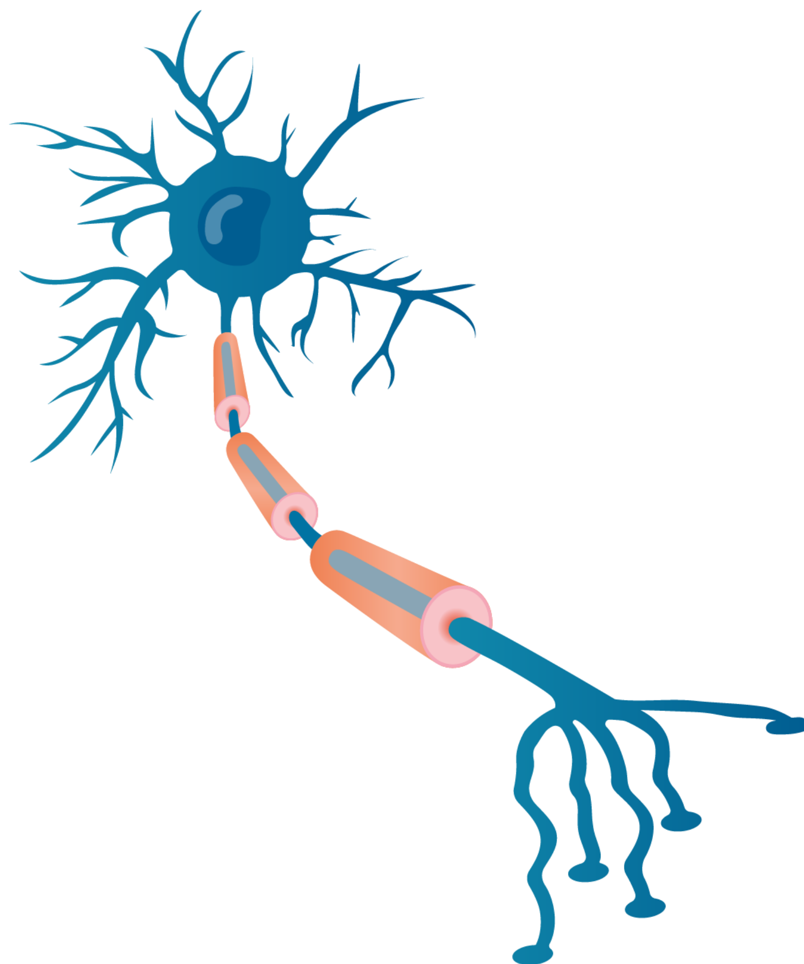
Postnatal axonal elimination is a regulated multireceptor mechanism involving the cooperation of several muscarinic and adenosine receptor subtypes during P7 and P9.

- A_1 by itself has the same effect in delaying the process as A_1 and A_{2A} have together at P7.
- The blockade of M_4 together with an M_1 or A_1 blockade is not different from the effect of blocking M_4 alone, hastening the process, but is significantly different from the individual effect of M_1 and A_1 at P7.
- M_2 receptor may modulate (by allowing a permissive action) the other receptors, mainly M_4 and A_1 at P7.
- M_1, A_1 and A_{2A} receptors cooperate to promote axonal loss at P9, whereas the potent axonal loss-promoting effect of M_2 is largely independent of the other receptors.
- There is a full mutual dependence between A_1 and A_{2A} receptors in regulating axon loss at P9.
- M_4 receptor (which in itself does not affect axon loss) modulates A_1, A_{2A} and M_2 receptors at P9 accelerating or hastening the process.

OBJECTIVE 5. To know the interaction between mAChRs (M_1, M_2 and M_4) and TrkB signaling in the modulation of the synapse elimination during development. To identify the number of motor nerve terminals per synapse at P9.

Postnatal axonal elimination is a regulated multireceptor mechanism involving the cooperation of several muscarinic subtypes and TrkB receptor during P9.

- M_1 and TrkB also contribute separately to axonal loss, but their combined action has a potent summed effect in accelerating the process similar to the effect of the M_2 receptor.
- M_2 has the strongest effect promoting axonal elimination and increases the axonal loss rate most with a slight involvement of the M_4 receptor but independently of the M_1 and TrkB receptors.
- M_4 regulates TrkB receptor producing a delay in the process whereas M_4 does not change M_1 effect on axonal loss.



VII. REFERENCES

Abbs ET, Joseph DN (1981) The effects of atropine and oxotremorine on acetylcholine release in rat phrenic nerve-diaphragm preparations. Br J Pharmacol 73:481–483.

Adámek S, Shakirzyanova A, Malomouzh A, Naumenko N, Vyskocil F (2010) Interaction of glutamate- and adenosine-induced decrease of acetylcholine quantal release at frog neuromuscular junction. Physiol Res 59:803–810.

Alicea D, Perez M, Maldonado C, Dominicci-Cotto C, Marie B (2017) Cortactin is a regulator of activity-dependent synaptic plasticity controlled by wingless. J Neurosci 37:2203–2215.

Allen DL, Leiter PA, Tielking RL, Hoffman WP, Vidyashankar AN, van Lier RBL, Wolff RK (1999) Effects of inhalation exposures to an M_1-receptor agonist on ventilation in rhesus monkeys. Drug Chem Toxicol 22:595–611.

Amaral MD, Pozzo-Miller L (2012) Intracellular Ca^{2+} stores and Ca^{2+} influx are both required for BDNF to rapidly increase quantal vesicular transmitter release. Neural Plast 2012:203536.

Andlauer TFM, Sigrist SJ (2012) *In vivo* imaging of Drosophila larval neuromuscular junctions to study synapse assembly. Cold Spring Harb Protoc 4:68577.

Angaut-Petit D, Molgo J, Connold AL, Faille L (1987) The *Levator auris longus* muscle of the mouse: a convenient preparation for studies of short- and long-term presynaptic effects of drugs or toxins. Neurosci Lett 82:83–88.

Angeli P, Cantalamessa F, Gulini U, Melchiorre C (1995) Selective blockade of muscarinic M_2 receptors *in vivo* by the new antagonist tripitramine. Naunyn Schmiedebergs Arch Pharmacol 352:304–307.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208–215.

Arellano RO, Garay E, Miledi R (1999) Muscarinic receptor heterogeneity in follicle-enclosed Xenopus oocytes. J Physiol 2:409–419.

Arenson MS (1989) Muscarinic inhibition of quantal transmitter release from the magnesium-paralysed frog sartorius muscle. Neuroscience 30:827–836.

Arevalo JC, Pereira DB, Yano H, Teng KK, Chao MV (2006) Identification of a switch in neurotrophin signaling by selective tyrosine phosphorylation. J Biol Chem 281:1001–1007.

Auld DS, Colomar A, Bélair E-L, Castonguay A, Pinard A, Rousse I, Thomas S, Robitaille R (2003) Modulation of neurotransmission by reciprocal synapse-glial interactions at the neuromuscular junction. J Neurocytol 32:1003–1015.

Auld DS, Robitaille R (2003) Perisynaptic Schwann cells at the neuromuscular junction: nerve- and activity-dependent contributions to synaptic efficacy, plasticity, and reinnervation. Neurosci 9:144–157.

Bairam A, Joseph V, Lajeunesse Y, Kinkead R (2006) Developmental pattern of M_1 and M_2 muscarinic gene expression and receptor levels in cat carotid body, petrosal and superior cervical ganglion. Neuroscience 139:711–721.

Bajjalieh SM, Scheller RH (1995) The biochemistry of neurotransmitter secretion. J Biol Chem 270:1971–1974.

Baldwin JE, Shiau CY, Byford MF, Schofield CJ (1994) Substrate specificity of L-delta-(alpha-aminoadipoyl)-L-cysteinyl-D-valine synthetase from *Cephalosporium acremonium*: demonstration of the structure of several unnatural tripeptide products. Biochem J:367–372.

Balice-Gordon RJ, Lichtman JW (1990) *In vivo* visualization of the growth of pre- and postsynaptic elements of neuromuscular junctions in the mouse. J Neurosci 10:894–908.

Balice-Gordon RJ, Lichtman JW (1993) *In vivo* observations of pre- and postsynaptic changes during the transition from multiple to single innervation at developing neuromuscular junctions. J Neurosci 13:834–855.

Baraldi PG, Tabrizi MA, Preti D, Bovero A, Romagnoli R, Fruttarolo F, Zaid NA, Moorman AR, Varani K, Gessi S, Merighi S, Borea PA (2004) Design, synthesis, and biological evaluation of new 8-heterocyclic xanthine derivatives as highly potent and selective human A_{2B} adenosine receptor antagonists. J Med Chem 47:1434–1447.

Bartoli M, Ramarao MK, Cohen JB (2001) Interactions of the rapsyn RING-H2 domain with dystroglycan. J Biol Chem 276:24911–24917.

Bauer CS, Woolley RJ, Teschemacher AG, Seward EP (2007) Potentiation of exocytosis by phospholipase C-coupled G-protein-coupled receptors requires the priming protein Munc13-1. J Neurosci 27:212–219.

Baumgold J, Nikodijevic O, Jacobson KA (1992) Penetration of adenosine antagonists into mouse brain as determined by *ex vivo* binding. Biochem Pharmacol 43:889–894.

Baysinger AN, Kent BA, Brown TH (2012) Muscarinic receptors in amygdala control trace fear conditioning. PLoS One 7:e45720.

Bebbington A, Brimblecombe RW, Shakeshaft D (1966) The central and peripheral activity of acetylenic amines related to oxotremorine. Br J Pharmacol Chemother 26:56–67.

Belluardo N, Westerblad H, Mudó G, Casabona A, Bruton J, Caniglia G, Pastoris O, Grassi F, Ibáñez CF (2001) Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4. Mol Cell Neurosci 18:56–67.

Benoit P, Changeux JP (1975) Consequences of tenotomy on the evolution of multiinnervation in developing rat soleus muscle. Brain Res 99:354–358.

Bernstein M, Lichtman JW (1999) Axonal atrophy: the retraction reaction. Curr Opin Neurobiol 9:364–370.

Besalduch N, Tomàs M, Santafé MM, Garcia N, Tomàs J, Lanuza MA (2010) Synaptic activity-related classical protein kinase C isoform localization in the adult rat neuromuscular synapse. J Comp Neurol 518:211–228.

Bewick GS, Young C, Slater CR (1996) Spatial relationships of utrophin, dystrophin, beta-dystroglycan and beta-spectrin to acetylcholine receptor clusters during postnatal maturation of the rat neuromuscular junction. J Neurocytol 25:367–379.

Bibel M, Barde YA (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 14:2919–2937.

Bishop DL, Misgeld T, Walsh MK, Gan W-B, Lichtman JW (2004) Axon branch removal at developing synapses by axosome shedding. Neuron 44:651–661.

Black JA, Kocsis JD, Waxman SG (1990) Ion channel organization of the myelinated fiber. Trends Neurosci 13:48–54.

Blitz DM, Pritchard AE, Latimer JK, Wakefield AT (2017) Muscles innervated by a single motor neuron exhibit divergent synaptic properties on multiple time scales. J Exp Biol:jeb.148908.

Bloch-Gallego E (2015) Mechanisms controlling neuromuscular junction stability. Cell Mol Life Sci 72:1029–1043.

Blöchl A, Thoenen H (1995) Characterization of nerve growth factor (NGF) release from hippocampal neurons: evidence for a constitutive and an unconventional sodium-dependent regulated pathway. Eur J Neurosci 7:1220–1228.

Bowe MA, Deyst KA, Leszyk JD, Fallon JR (1994) Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: a heteromeric complex related to the dystroglycans. Neuron 12:1173–1180.

Bowerman M, Beauvais A, Anderson CL, Kothary R (2010) Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. Hum Mol Genet 19:1468–1478.

Brand-Saberi B, Wilting J, Ebensperger C, Christ B (1996) The formation of somite compartments in the avian embryo. Int J Dev Biol 40:411–420.

Brenner HR, Sakmann B (1978) Gating properties of acetycholine receptor in newly formed neuromuscular synapses. Nature 271:366–368.

Brill MS, Lichtman JW, Thompson W, Zuo Y, Misgeld T (2011) Spatial constraints dictate glial territories at murine neuromuscular junctions. J Cell Biol 195:293–305.

Brown JH, Brown SL (1984) Agonists differentiate muscarinic receptors that inhibit cyclic AMP formation from those that stimulate phosphoinositide metabolism. J Biol Chem 259:3777–3781.

Brown MC, Jansen JK, Van Essen D (1976) Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. J Physiol 261:387–422.

Buffelli M, Busetto G, Cangiano L, Cangiano A (2002) Perinatal switch from synchronous to asynchronous activity of motoneurons: link with synapse elimination. Proc Natl Acad Sci U S A 99:13200–13205.

Buffelli M, Burgess RW, Feng G, Lobe CG, Lichtman JW, Sanes JR (2003) Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. Nature 424:430–434.

Buffelli M, Busetto G, Bidoia C, Favero M, Cangiano A (2004) Activity-dependent synaptic competition at mammalian neuromuscular junctions. News Physiol Sci 19:85–91.

Burden S (1977) Development of the neuromuscular junction in the chick embryo: the number, distribution, and stability of acetylcholine receptors. Dev Biol 57:317–329.

Burnstock G (2007) Purine and pyrimidine receptors. Cell Mol Life Sci 64:1471–1483.

Busetto G, Buffelli M, Tognana E, Bellico F, Cangiano A (2000) Hebbian mechanisms revealed by electrical stimulation at developing rat neuromuscular junctions. J Neurosci 20:685–695.

Campagna JA, Rüegg MA, Bixby JL (1995) Agrin is a differentiation-inducing "stop signal" for motoneurons *in vitro*. Neuron 15:1365–1374.

Caroni P (1997) Overexpression of growth-associated proteins in the neurons of adult transgenic mice. J Neurosci Methods 71:3–9.

Castrén E, Rantamäki T (2010) The role of BDNF and its receptors in depression and antidepressant drug action: reactivation of developmental plasticity. Dev Neurobiol 70:289–297.

Caulfield MP (1993) Muscarinic receptors-characterization, coupling and function. Pharmacol Ther 58 (3):319–379.

Caulfield MP, Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290.

Chambers TC, Pohl J, Glass DB, Kuo JF (1994) Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. Biochem J 299 (1):309–315.

Chang Q, Balice-Gordon RJ (1997) Nip and tuck at the neuromuscular junction: a role for proteases in developmental synapse elimination. BioEssays 19:271–275.

Chao M V. (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4:299–309.

Chen C, Regehr WG (2000) Developmental remodeling of the retinogeniculate synapse. Neuron 28:955–966.

Chen J, Tan Z, Zeng L, Zhang X, He Y, Gao W, Wu X, Li Y, Bu B, Wang W, Duan S (2013) Heterosynaptic long-term depression mediated by ATP released from astrocytes. Glia 61:178–191.

Chieng B, Bekkers JM (2001) Inhibition of calcium channels by opioid- and adenosine-receptor agonists in neurons of the nucleus accumbens. Br J Pharmacol 133:337–344.

Chow I, Poo MM (1985) Release of acetylcholine from embryonic neurons upon contact with muscle cell. J Neurosci 5:1076–1082.

Chwalczuk K, Rubaj A, Swiader M, Czuczwar SJ (2008) Influence of the antagonist of adenosine A_1 receptors, 8-cyclopentyl-1 ,3-dipropylxanthine, upon the anticonvulsant activity of antiepileptic drugs in mice. Przegl Lek 65:759–763.

Clark M, Finkel R, Rey R, Whalen K (2012) Pharmacology (Lippincott Illustrated Reviews Series), 6th ed. Baltimore: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Coates J, Sheehan MJ, Strong P (1994) 1,3-Dipropyl-8-cyclopentyl xanthine (DPCPX): a useful tool for pharmacologists and physiologists? Gen Pharmacol 25:387–394.

Cohen-Cory S (2002) The developing synapse: construction and modulation of synaptic structures and circuits. Science 298:770–776.

Cohen MV, Downey JM (2008) Adenosine: trigger and mediator of cardioprotection. Basic Res Cardiol 103:203–215.

Colman H, Nabekura J, Lichtman JW (1997) Alterations in synaptic strength preceding axon withdrawal. Science 275:356–361.

Corfas G, Velardez MO, Ko C-P, Ratner N, Peles E (2004) Mechanisms and roles of axon-Schwann cell interactions. J Neurosci 24:9250–9260.

Correia-de-Sá P, Sebastião AM, Ribeiro JA (1991) Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. Br J Pharmacol 103:1614–1620.

Cossu G, Biressi S (2005) Satellite cells, myoblasts and other occasional myogenic progenitors: possible origin, phenotypic features and role in muscle regeneration. Semin Cell Dev Biol 16:623–631.

Costenla AR, Cunha RA, de Mendonça A (2010) Caffeine, adenosine receptors, and synaptic plasticity. J Alzheimer's Dis 20:S25-34.

Court FA, Gillingwater TH, Melrose S, Sherman DL, Greenshields KN, Morton AJ, Harris JB, Willison HJ, Ribchester RR (2008) Identity, developmental restriction and reactivity of extralaminar cells capping mammalian neuromuscular junctions. J Cell Sci 121:3901–3911.

Courteaux R (1973) Motor endplate structure. In structure and function of muscle (GH B, ed). New York: Academic.

Croll SD, Chesnutt CR, Rudge JS, Acheson A, Ryan TE, Siuciak JA, DiStefano PS, Wiegand SJ, Lindsay RM (1998) Co-infusion with a TrkB-Fc receptor body carrier enhances BDNF distribution in the adult rat brain. Exp Neurol 152:20–33.

Cunha C, Brambilla R, Thomas KL (2010) A simple role for BDNF in learning and memory? Front Mol Neurosci 3:1.

Dai C, Liu N, Chen W, Qian W, Hou X (2012) Simo decoction promotes contraction of antral circular smooth muscle mainly via muscarinic M_3 receptor. J Ethnopharmacol 144:270–276.

Daly JW, Padgett W, Shamim MT, Butts-Lamb P, Waters J (1985) 1,3-Dialkyl-8-(p-sulfophenyl)xanthines: potent water-soluble antagonists for A_1- and A_2-adenosine receptors. J Med Chem 28:487–492.

Daniel H, Hemart N, Jaillard D, Crepel F (1992) Coactivation of metabotropic glutamate receptors and of voltage-gated calcium channels induces long-term depression in cerebellar Purkinje cells *in vitro*. Exp brain Res 90:327–331.

Davies KE, Nowak KJ (2006) Molecular mechanisms of muscular dystrophies: old and new players. Nat Rev Mol Cell Biol 7:762–773.

Day NC, Wood SJ, Ince PG, Volsen SG, Smith W, Slater CR, Shaw PJ (1997) Differential localization of voltage-dependent calcium channel alpha1 subunits at the human and rat neuromuscular junction. J Neurosci 17:6226–6235.

De Lange RP, De Roos AD, Borst JG (2003) Two modes of vesicle recycling in the rat calyx of Held. J Neurosci 23:10164–10173.

De Lera Ruiz M, Lim Y-H, Zheng J (2014) Adenosine A_{2A} receptor as a drug discovery target. J Med Chem 57:3623–3650.

De Lorenzo S, Veggetti M, Muchnik S, Losavio A (2006) Presynaptic inhibition of spontaneous acetylcholine release mediated by P2Y receptors at the mouse neuromuscular junction. Neuroscience 142:71–85.

De Mendonça A, Almeida T, Bashir ZI, Ribeiro JA (1997) Endogenous adenosine attenuates long-term depression and depotentiation in the CA1 region of the rat hippocampus. Neuropharmacology 36:161–167.

De Sarro G, Donato Di Paola E, Falconi U, Ferreri G, De Sarro A (1996) Repeated treatment with adenosine A_1 receptor agonist and antagonist modifies the anticonvulsant properties of CPPene. Eur J Pharmacol 317:239–245.

Dechant G, Barde Y-A (2002) The neurotrophin receptor p75NTR: novel functions and implications for diseases of the nervous system. Nat Neurosci 5:1131–1136.

De Chiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation *in vivo*. Cell 85:501–512.

Del Castillo J, Katz B (1954) Quantal components of the end-plate potential. J Physiol 124:560–573.

Delgado R, Maureira C, Oliva C, Kidokoro Y, Labarca P (2000) Size of vesicle pools, rates of mobilization, and recycling at neuromuscular synapses of a Drosophila mutant, shibire. Neuron 28:941–953.

Della Latta V, Cabiati M, Rocchiccioli S, Del Ry S, Morales M-A (2013) The role of the adenosinergic system in lung fibrosis. Pharmacol Res 76:182–189.

Denker A, Bethani I, Krohnert K, Korber C, Horstmann H, Wilhelm BG, Barysch SV, Kuner T, Neher E, Rizzoli SO (2011) A small pool of vesicles maintains synaptic activity *in vivo*. Proc Natl Acad Sci 108:17177–17182.

Dennis MJ (1981) Development of the neuromuscular junction: inductive interactions between cells. Annu Rev Neurosci 4:43–68.

Deschenes MR, Maresh CM, Armstrong LE, Covault J, Kraemer WJ, Crivello JF (1994) Endurance and resistance exercise induce muscle fiber type specific responses in androgen binding capacity. J Steroid Biochem Mol Biol 50:175–179.

Dias RB, Rombo DM, Ribeiro JA, Henley JM, Sebastião AM (2013) Adenosine: setting the stage for plasticity. Trends Neurosci 36:248–257.

Donato R, Canepari M, Lape R, Nistri A (2003) Effects of caffeine on the excitability and intracellular Ca^{2+} transients of neonatal rat hypoglossal motoneurons *in vitro*. Neurosci Lett 346:177–181.

Duclert A, Changeux JP (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. Physiol Rev 75:339–368.

Duxson MJ (1982) The effect of postsynaptic block on development of the neuromuscular junction in postnatal rats. J Neurocytol 11:395–408.

Eaton BA, Fetter RD, Davis GW (2002) Dynactin is necessary for synapse stabilization. Neuron 34:729–741.

El Yacoubi M, Ledent C, Parmentier M, Ongini E, Costentin J, Vaugeois JM (2001) *In vivo* labelling of the adenosine A_{2A} receptor in mouse brain using the selective antagonist (3H)SCH 58261. Eur J Neurosci 14:1567–1570.

Engel A (1994a) The neuromuscular junction. In myology: Basic and Clinical (AG E, ed)., 1st ed. New York: McGraw-Hill.

Engel A (1994b) The molecular biology of neuronal nicotinic acetylcholine receptors (AG E, ed).

Engel A (1994c) Nicotinic acetylcholine receptors: from structure to brain function (AG E, ed). Berlin, Heidelberg: Springer Berlin Heidelberg.

Engel A, Franzini-Armstrong C (2004) The neuromuscular junction. In myology: Basic and Clinical (C EA and F-A, ed). New York: McGraw-Hill.

Ervasti JM, Campbell KP (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J Cell Biol 122:809–823.

Erzen I, Cvetko E, Obreza S, Angaut-Petit D (2000) Fiber types in the mouse *Levator auris longus* muscle: A convenient preparation to study muscle and nerve plasticity. J Neurosci Res 59:692–697.

Evers J, Laser M, Sun YA, Xie ZP, Poo MM (1989) Studies of nerve-muscle interactions in Xenopus cell culture: analysis of early synaptic currents. J Neurosci 9:1523–1539.

Fatt P, Katz B (1952) The electric activity of the motor end-plate. Proc R Soc London Ser B, Biol Sci 140:183–186.

Favero M, Massella O, Cangiano A, Buffelli M (2009) On the mechanism of action of muscle fibre activity in synapse competition and elimination at the mammalian neuromuscular junction. Eur J Neurosci 29:2327–2334.

Favero M, Busetto G, Cangiano A (2012) Spike timing plays a key role in synapse elimination at the neuromuscular junction. Proc Natl Acad Sci U S A 109:E1667-75.

Favero M, Cangiano A, Busetto G (2015) Adult rat motor neurons do not re-establish electrical coupling during axonal regeneration and muscle reinnervation. PLoS One 10:e0123576.

Federico S, Spalluto G (2012) Therapeutic potential of A_2 and A_3 adenosine receptor: a review of novel patented ligands. Expert Opin Ther Pat 22:369–390.

Felder CC (1995) Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J 9:619–625.

Feng G, Krejci E, Molgo J, Cunningham JM, Massoulié J, Sanes JR (1999) Genetic analysis of collagen Q: roles in acetylcholinesterase and butyrylcholinesterase assembly and in synaptic structure and function. J Cell Biol 144:1349–1360.

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41–51.

Feng Z, Ko C-P (2008) The role of glial cells in the formation and maintenance of the neuromuscular junction. Ann N Y Acad Sci 1132:19–28.

Ferré S, Popoli P, Rimondini R, Reggio R, Kehr J, Fuxe K (1999) Adenosine A_{2A} and group I metabotropic glutamate receptors synergistically modulate the binding characteristics of dopamine D_2 receptors in the rat striatum. Neuropharmacology 38:129–140.

Fertuck HC, Salpeter MM (1974) Localization of acetylcholine receptor by 125I-labeled alpha-bungarotoxin binding at mouse motor endplates. Proc Natl Acad Sci U S A 71:1376–1378.

Fisher A, Brandeis R, Bar-Ner RHN, Kliger-Spatz M, Natan N, Sonego H, Marcovitch I, Pittel Z (2002) AF150(S) and AF267B: M_1 muscarinic agonists as innovative therapies for Alzheimer's disease. J Mol Neurosci 19:145–153.

Fisher SK, Figueiredo JC, Bartus RT (1984) Differential stimulation of inositol phospholipid turnover in brain by analogs of oxotremorine. J Neurochem 43:1171–1179.

Fladby T, Jansen JK (1988) Selective innervation of neonatal fast and slow muscle fibres before net loss of synaptic terminals in the mouse soleus muscle. Acta Physiol Scand 134:561–562.

Flucher BE, Daniels MP (1989) Distribution of Na^+ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and the 43 kd protein. Neuron 3:163–175.

Fredholm BB, Irenius E, Kull B, Schulte G (2001) Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. Biochem Pharmacol 61:443–448.

Funakoshi H, Frisén J, Barbany G, Timmusk T, Zachrisson O, Verge VM, Persson H (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J Cell Biol 123:455–465.

Funakoshi H, Belluardo N, Arenas E, Yamamoto Y, Casabona A, Persson H, Ibáñez CF (1995) Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. Science 268:1495–1499.

Fuxe K, Marcellino D, Genedani S, Agnati L (2007) Adenosine A_{2A} receptors, dopamine D_2 receptors and their interactions in Parkinson's disease. Mov Disord 22:1990–2017.

Gall CM, Isackson PJ (1989) Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. Science 245:758–761.

Gamberini MT, Bolognesi ML, Nasello AG (2012) The modulatory role of M_2 muscarinic receptor on apomorphine-induced yawning and genital grooming. Neurosci Lett 531:91–95.

Gan WB, Lichtman JW (1998) Synaptic segregation at the developing neuromuscular junction. Science 282:1508–1511.

Ganguly DK, Das M (1979) Effects of oxotremorine demonstrate presynaptic muscarinic and dopaminergic receptors on motor nerve terminals. Nature 278:645–646.

Garcia N, Santafé MM, Salon I, Lanuza MA, Tomàs J (2005) Expression of muscarinic

acetylcholine receptors (M_1-, M_2-, M_3- and M_4-type) in the neuromuscular junction of the newborn and adult rat. Histol Histopathol 20:733–743.

Garcia N, Santafé MM, Tomàs M, Lanuza MA, Besalduch N, Priego M, Tomàs J (2010a) The glial cell line-derived neurotrophic factor (GDNF) does not acutely change acetylcholine release in developing and adult neuromuscular junction. Neurosci Lett 480:127–131.

Garcia N, Santafé MM, Tomas M, Lanuza MA, Besalduch N, Tomas J (2010b) Neurotrophin-4 couples to locally modulated ACh release at the end of neuromuscular synapse maturation. Neurosci Lett 468:72–74.

Garcia N, Santafé MM, Tomàs M, Lanuza MA, Besalduch N, Tomàs J (2010c) Involvement of neurotrophin-3 (NT-3) in the functional elimination of synaptic contacts during neuromuscular development. Neurosci Lett 473:141–145.

Garcia N, Santafé MM, Tomàs M, Lanuza MA, Besalduch N, Tomàs J (2010d) Involvement of brain-derived neurotrophic factor (BDNF) in the functional elimination of synaptic contacts at polyinnervated neuromuscular synapses during development. J Neurosci Res 88:NA-NA.

Garcia N, Tomàs M, Santafé MM, Besalduch N, Lanuza MA, Tomàs J (2010e) The interaction between tropomyosin-related kinase B receptors and presynaptic muscarinic receptors modulates transmitter release in adult rodent motor nerve terminals. J Neurosci 30:16514–16522.

Garcia N, Tomàs M, Santafé MM, Lanuza MA, Besalduch N, Tomàs J (2010f) Localization of brain-derived neurotrophic factor, neurotrophin-4, tropomyosin-related kinase b receptor, and p75 NTR receptor by high-resolution immunohistochemistry on the adult mouse neuromuscular junction. J Peripher Nerv Syst 15:40–49.

Garcia N, Tomàs M, Santafé MM, Lanuza MA, Besalduch N, Tomàs J (2011) Blocking p75 (NTR) receptors alters polyinnervationz of neuromuscular synapses during development. J Neurosci Res 89:1331–1341.

Garcia N, Priego M, Obis T, Santafe MM, Tomàs M, Besalduch N, Lanuza MA, Tomàs J (2013) Adenosine A_1 and A_{2A} receptor-mediated modulation of acetylcholine release in the mice neuromuscular junction. Eur J Neurosci 38:2229–2241.

Garcia N, Priego M, Hurtado E, Obis T, Santafé MM, Tomàs M, Lanuza MA, Tomàs J (2014) Adenosine A_{2B} and A_3 receptor location at the mouse neuromuscular junction. J Anat 225:109–117.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 85:525–535.

Georgiou J, Robitaille R, Charlton MP (1999) Muscarinic control of cytoskeleton in perisynaptic glia. J Neurosci 19:3836–3846.

Giglio D, Aronsson P, Eriksson L, Tobin G (2007) *In vitro* characterization of parasympathetic and sympathetic responses in cyclophosphamide-induced cystitis in the rat. Basic Clin Pharmacol Toxicol 100:96–108.

Gonzalez M, Ruggiero FP, Chang Q, Shi YJ, Rich MM, Kraner S, Balice-Gordon RJ (1999) Disruption of TrkB-mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. Neuron 24:567–583.

Goodman L, Brunton L, Chabner B, Knollmann B (2011) Goodman & Gilman's pharmacological basis of therapeutics. New York: McGraw-Hill.

Gordon JW, Chesa PG, Nishimura H, Rettig WJ, Maccari JE, Endo T, Seravalli E, Seki T, Silver J (1987) Regulation of Thy-1 gene expression in transgenic mice. Cell 50:445–452.

Gregory K, Sexton P, Christopoulos A (2007) Allosteric modulation of muscarinic acetylcholine receptors. Curr Neuropharmacol 5:157–167.

Gustafsson E, Lindvall O, Kokaia Z (2003) Intraventricular infusion of TrkB-Fc fusion protein promotes ischemia-induced neurogenesis in adult rat dentate gyrus. Stroke 34:2710–2715.

Harvey RD (2012) Muscarinic receptor agonists and antagonists: effects on cardiovascular function. In: Handbook of experimental pharmacology, pp 299–316.

Hashimoto K, Kano M (2005) Postnatal development and synapse elimination of climbing fiber to Purkinje cell projection in the cerebellum. Neurosci Res 53:221–228.

Haskó G, Pacher P, Sylvester Vizi E, Illes P (2005) Adenosine receptor signaling in the brain immune system. Trends Pharmacol Sci 26:511–516.

He X, Semenov M, Tamai K, Zeng X (2004) LDL receptor-related proteins 5 and 6 in Wnt/β-catenin signaling: Arrows point the way. Development 131:1663–1677.

Hebb D (1949) The organization of behavior. New York: Wiley.

Herbst R, Burden SJ (2000) The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling. EMBO J 19:67–77.

Herbst R, Avetisova E, Burden SJ (2002) Restoration of synapse formation in Musk mutant mice expressing a Musk/Trk chimeric receptor. Development 129:5449–5460.

Herrera AA, Zeng Y (2003) Activity-dependent switch from synapse formation to synapse elimination during development of neuromuscular junctions. J Neurocytol 32:817–833.

Heuser JE, Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315–344.

Hirata K, Zhou C, Nakamura K, Kawabuchi M (1997) Postnatal development of Schwann cells at neuromuscular junctions, with special reference to synapse elimination. J Neurocytol 26:799–809.

Hirsch NP (2007) Neuromuscular junction in health and disease. Br J Anaesth 99:132–138.

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677–736.

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609–642.

Hubbard SR, Gnanasambandan K (2013) Structure and activation of MuSK, a receptor tyrosine kinase central to neuromuscular junction formation. Biochim Biophys Acta 1834:2166–2169.

Hubel DH, Wiesel TN, LeVay S (1977) Plasticity of ocular dominance columns in monkey striate cortex. Philos Trans R Soc Lond B Biol Sci 278:377–409.

Hume RI, Role LW, Fischbach GD (1983) Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. Nature 305:632–634.

Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 355:696–702.

Ikeda K, Bekkers JM (2009) Counting the number of releasable synaptic vesicles in a presynaptic terminal. Proc Natl Acad Sci U S A 106:2945–2950.

Ip FC, Cheung J, Ip NY (2001) The expression profiles of neurotrophins and their receptors in rat and chicken tissues during development. Neurosci Lett 301:107–110.

Ivan HK and Etherington SH (2011) Neuromuscular junction. Chichester, UK: John Wiley & Sons Ltd.

Jahn R, Fasshauer D (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490:201–207.

Jakubík J, El-Fakahany EE (2010) Allosteric modulation of muscarinic acetylcholine receptors. Pharmaceuticals 3:2838–2860.

Jansen JK, Fladby T (1990) The perinatal reorganization of the innervation of skeletal muscle in mammals. Prog Neurobiol 34:39–90.

Jaramillo F, Vicini S, Schuetze SM (1988) Embryonic acetylcholine receptors guarantee spontaneous contractions in rat developing muscle. Nature 335:66–68.

Je HS, Yang F, Ji Y, Nagappan G, Hempstead BL, Lu B (2012) Role of pro-brain-derived neurotrophic factor (proBDNF) to mature BDNF conversion in activity-dependent competition at developing neuromuscular synapses. Proc Natl Acad Sci U S A 109:15924–15929.

Je HS, Yang F, Ji Y, Potluri S, Fu X-Q, Luo Z-G, Nagappan G, Chan JP, Hempstead B, Son Y-J, Lu B (2013) ProBDNF and mature BDNF as punishment and reward signals for synapse elimination at mouse neuromuscular junctions. J Neurosci 33:9957–9962.

Jennings C (1994) Developmental neurobiology. Death of a synapse. Nature 372:498–499.

Jessen KR, Mirsky R (2005) The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci 6:671–682.

Jia M, Li M, Dunlap V, Nelson PG (1999) The thrombin receptor mediates functional activity-dependent neuromuscular synapse reduction via protein kinase C activation *in vitro*. J Neurobiol 38:369–381.

Jing L, Lefebvre JL, Gordon LR, Granato M (2009) Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. Neuron 61:721–733.

Jolkkonen M, van Giersbergen PL, Hellman U, Wernstedt C, Karlsson E (1994) A toxin from the green mamba Dendroaspis angusticeps: amino acid sequence and

selectivity for muscarinic M_4 receptors. FEBS Lett 352:91–94.

Kang H, Tian L, Thompson W (2003) Terminal Schwann cells guide the reinnervation of muscle after nerve injury. J Neurocytol 32:975–985.

Kano M, Hashimoto K (2009) Synapse elimination in the central nervous system. Curr Opin Neurobiol 19:154–161.

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10:381–391.

Keller-Peck CR, Feng G, Sanes JR, Yan Q, Lichtman JW, Snider WD (2001) Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. J Neurosci 21:6136–6146.

Kelley KA, Friedrich VL, Sonshine A, Hu Y, Lax J, Li J, Drinkwater D, Dressler H, Herrup K (1994) Expression of Thy-1/lacZ fusion genes in the CNS of transgenic mice. Brain Res Mol Brain Res 24:261–274.

Kemp N, Bashir ZI (1997) A role for adenosine in the regulation of long-term depression in the adult rat hippocampus *in vitro*. Neurosci Lett 225:189–192.

Keshishian H, Broadie K, Chiba A, Bate M (1996) The Drosophila neuromuscular junction: a model system for studying synaptic development and function. Annu Rev Neurosci 19:545–575.

Kettenmann H, Ransom BR (2005) Neuroglia (Kettenmann H and Ransom BR, ed)., 2nd ed. New York: Oxford University Press.

Kidokoro Y, Yeh E (1982) Initial synaptic transmission at the growth cone in Xenopus nerve-muscle cultures. Proc Natl Acad Sci U S A 79:6727–6731.

Kim DS, Palmiter RD (2008) Interaction of dopamine and adenosine receptor function in behavior: studies with dopamine-deficient mice. Front Biosci 13:2311–2318.

Kim N, Burden SJ (2008) MuSK controls where motor axons grow and form synapses. Nat Neurosci 11:19–27.

Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, Hubbard SR, Dustin ML, Burden SJ (2008) Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell 135:334–342.

Klinkenberg I, Sambeth A, Blokland A (2013) Cholinergic gating of hippocampal auditory evoked potentials in freely moving rats. Eur Neuropsychopharmacol 23:988–997.

Ko C-P, Robitaille R (2015) Perisynaptic Schwann cells at the neuromuscular synapse: adaptable, multitasking glial cells. Cold Spring Harb Perspect Biol 7:a020503.

Koirala S, Ko C-P (2004) Pruning an axon piece by piece: a new mode of synapse elimination. Neuron 44:578–580.

Kollias G, Spanopoulou E, Grosveld F, Ritter M, Beech J, Morris R (1987) Differential regulation of a Thy-1 gene in transgenic mice. Proc Natl Acad Sci U S A 84:1492–1496.

Kopp DM, Perkel DJ, Balice-Gordon RJ (2000) Disparity in neurotransmitter release

probability among competing inputs during neuromuscular synapse elimination. J Neurosci 20:8771–8779.

Krejci E, Thomine S, Boschetti N, Legay C, Sketelj J, Massoulié J (1997) The mammalian gene of acetylcholinesterase-associated collagen. J Biol Chem 272:22840–22847.

Kudlacek O, Just H, Korkhov VM, Vartian N, Klinger M, Pankevych H, Yang Q, Nanoff C, Freissmuth M, Boehm S (2003) The human D_2 dopamine receptor synergizes with the A_{2A} adenosine receptor to stimulate adenylyl cyclase in PC12 cells. Neuropsychopharmacology 28:1317–1327.

Kues WA, Sakmann B, Witzemann V (1995) Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. Eur J Neurosci 7:1376–1385.

Kullberg RW, Lentz TL, Cohen MW (1977) Development of the myotomal neuromuscular junction in Xenopus laevis: an electrophysiological and fine-structural study. Dev Biol 60:101–129.

Kumari A, Sreetama S, Mohanakumar KP (2007) Atropine, a muscarinic cholinergic receptor antagonist increases serotonin, but not dopamine levels in discrete brain regions of mice. Neurosci Lett 423:100–103.

Kuromi H, Kidokoro Y (2000) Tetanic stimulation recruits vesicles from reserve pool via a cAMP-mediated process in Drosophila synapses. Neuron 27:133–143.

Kurowski P, Gawlak M, Szulczyk P (2015) Muscarinic receptor control of pyramidal neuron membrane potential in the medial prefrontal cortex (mPFC) in rats. Neuroscience 303:474–488.

Kwon YW, Gurney ME (1996) Brain-derived neurotrophic factor transiently stabilizes silent synapses on developing neuromuscular junctions. J Neurobiol 29:503–516.

Lähteinen S, Pitkänen A, Saarelainen T, Nissinen J, Koponen E, Castrén E (2002) Decreased BDNF signalling in transgenic mice reduces epileptogenesis. Eur J Neurosci 15:721–734.

Langmead CJ, Watson J, Reavill C (2008) Muscarinic acetylcholine receptors as CNS drug targets. Pharmacol Ther 117:232–243.

Lanuza MA, Garcia N, Santafé M, Nelson PG, Fenoll-Brunet MR, Tomas J (2001) Pertussis toxin-sensitive G-protein and protein kinase C activity are involved in normal synapse elimination in the neonatal rat muscle. J Neurosci Res 63:330–340.

Lanuza MA, Garcia N, Santafé M, González CM, Alonso I, Nelson PG, Tomàs J (2002) Pre- and postsynaptic maturation of the neuromuscular junction during neonatal synapse elimination depends on protein kinase C. J Neurosci Res 67:607–617.

Lanuza MA, Garcia N, González CM, Santafé MM, Nelson PG, Tomas J (2003) Role and expression of thrombin receptor PAR-1 in muscle cells and neuromuscular junctions during the synapse elimination period in the neonatal rat. J Neurosci Res 73:10–21.

Lanuza MA, Besalduch N, González C, Santafé MM, Garcia N, Tomàs M, Nelson PG, Tomàs J (2010) Decreased phosphorylation of δ and ε subunits of the acetylcholine receptor coincides with delayed postsynaptic maturation in PKC θ deficient mouse. Exp Neurol 225:183–195.

Lanuza MA, Santafe MM, Garcia N, Besalduch N, Tomàs M, Obis T, Priego M, Nelson PG, Tomàs J (2014) Protein kinase C isoforms at the neuromuscular junction: localization and specific roles in neurotransmission and development. J Anat 224:61–73.

Leber SM, Breedlove SM, Sanes JR (1990) Lineage, arrangement, and death of clonally related motoneurons in chick spinal cord. J Neurosci 10:2451–2462.

Lee F, Chao M (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc Natl Acad Sci 98:3555–3560.

Lee YI, Li Y, Mikesh M, Smith I, Nave K-A, Schwab MH, Thompson WJ (2016) Neuregulin1 displayed on motor axons regulates terminal Schwann cell-mediated synapse elimination at developing neuromuscular junctions. Proc Natl Acad Sci U S A 113:E479-87.

Li MX, Jia M, Yang LX, Jiang H, Lanuza MA, Gonzalez CM, Nelson PG (2004) The role of the theta isoform of protein kinase C (PKC) in activity-dependent synapse elimination: evidence from the PKC theta knock-out mouse *in vivo* and *in vitro*. J Neurosci 24:3762–3769.

Lichtman JW (1977) The reorganization of synaptic connexions in the rat submandibular ganglion during post-natal development. J Physiol 273:155–177.

Lichtman JW, Colman H (2000) Synapse elimination and indelible memory. Neuron 25:269–278.

Lin S, Landmann L, Ruegg MA, Brenner HR (2008) The role of nerve- versus muscle-derived factors in mammalian neuromuscular junction formation. J Neurosci 28:3333–3340.

Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee K-F (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature 410:1057–1064.

Linden DC, Jerian SM, Letinsky MS (1988) Neuromuscular junction development in the cutaneous pectoris muscle of Rana catesbeiana. Exp Neurol 99:735–760.

Liu Y, Fields RD, Festoff BW, Nelson PG (1994) Proteolytic action of thrombin is required for electrical activity-dependent synapse reduction. Proc Natl Acad Sci U S A 91:10300–10304.

Loeb JA, Hmadcha A, Fischbach GD, Land SJ, Zakarian VL (2002) Neuregulin expression at neuromuscular synapses is modulated by synaptic activity and neurotrophic factors. J Neurosci 22:2206–2214.

Lohof AM, Delhaye-Bouchaud N, Mariani J (1996) Synapse elimination in the central nervous system: functional significance and cellular mechanisms. Rev Neurosci 7:85–101.

Lohse MJ, Klotz KN, Lindenborn-Fotinos J, Reddington M, Schwabe U, Olsson RA (1987) 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)--a selective high affinity antagonist radioligand for A_1 adenosine receptors. Naunyn Schmiedebergs Arch Pharmacol 336:204–210.

Lu B (2003) BDNF and activity-dependent synaptic modulation. Learn Mem 10:86–98.

Luo ZG, Wang Q, Zhou JZ, Wang J, Luo Z, Liu M, He X, Wynshaw-Boris A, Xiong WC, Lu B,

Mei L (2002) Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. Neuron 35:489–505.

Mantilla CB, Zhan W-Z, Sieck GC (2004) Neurotrophins improve neuromuscular transmission in the adult rat diaphragm. Muscle Nerve 29:381–386.

Mantilla CB, Gransee HM, Zhan W-Z, Sieck GC (2013) Motoneuron BDNF/TrkB signaling enhances functional recovery after cervical spinal cord injury. Exp Neurol 247:101–109.

Marek GJ (2009) Activation of adenosine1 (A_1) receptors suppresses head shakes induced by a serotonergic hallucinogen in rats. Neuropharmacology 56:1082–1087.

Marques MJ, Conchello JA, Lichtman JW (2000) From plaque to pretzel: fold formation and acetylcholine receptor loss at the developing neuromuscular junction. J Neurosci 20:3663–3675.

Martinson EA, Johnson RA, Wells JN (1987) Potent adenosine receptor antagonists that are selective for the A_1 receptor subtype. Mol Pharmacol 31:247–252.

Martos F, Monferini E, Giraldo E, De Paoli AM, Hammer R (1987) Characterization of muscarinic receptors in salivary and lacrimal glands of the rat. Eur J Pharmacol 143:189–194.

Mashima R, Hishida Y, Tezuka T, Yamanashi Y (2009) The roles of Dok family adapters in immunoreceptor signaling. Immunol Rev 232:273–285.

Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, Metzger D, Reggiani C, Schiaffino S, Sandri M (2009) Autophagy is required to maintain muscle mass. Cell Metab 10:507–515.

Matthews-Bellinger JA, Salpeter MM (1983) Fine structural distribution of acetylcholine receptors at developing mouse neuromuscular junctions. J Neurosci 3:644–657.

McCool BA, Farroni JS (2001) A_1 adenosine receptors inhibit multiple voltage-gated Ca^{2+} channel subtypes in acutely isolated rat basolateral amygdala neurons. Br J Pharmacol 132:879–888.

McKinnon LA, Nathanson NM (1995) Tissue-specific regulation of muscarinic acetylcholine receptor expression during embryonic development. J Biol Chem 270:20636–20642.

Mei L, Nave K-A (2014) Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. Neuron 83:27–49.

Mei YA, Le Foll F, Vaudry H, Cazin L (1996) Adenosine inhibits L- and N-type calcium channels in pituitary melanotrophs. Evidence for the involvement of a G protein in calcium channel gating. J Neuroendocrinol 8:85–91.

Meier T, Masciulli F, Moore C, Schoumacher F, Eppenberger U, Denzer AJ, Jones G, Brenner HR (1998) Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. J Cell Biol 141:715–726.

Migita H, Kominami K, Higashida M, Maruyama R, Tuchida N, McDonald F, Shimada F, Sakurada K (2008) Activation of adenosine A_1 receptor-induced neural stem cell proliferation via MEK/ERK and Akt signaling pathways. J Neurosci Res 86:2820–

2828.

Minic J, Molgó J, Karlsson E, Krejci E (2002) Regulation of acetylcholine release by muscarinic receptors at the mouse neuromuscular junction depends on the activity of acetylcholinesterase. Eur J Neurosci 15:439–448.

Mirsky R, Jessen KR (1996) Schwann cell development, differentiation and myelination. Curr Opin Neurobiol 6:89–96.

Mis K, Matkovic U, Pirkmajer S, Sciancalepore M, Lorenzon P, Mars T, Grubic Z (2013) Acetylcholinesterase and agrin: different functions, similar expression patterns, multiple roles. Chem Biol Interact 203:297–301.

Misgeld T, Burgess RW, Lewis RM, Cunningham JM, Lichtman JW, Sanes JR (2002) Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. Neuron 36:635–648.

Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321:406–411.

Monopoli A, Lozza G, Forlani A, Mattavelli A, Ongini E (1998) Blockade of adenosine A_{2A} receptors by SCH 58261 results in neuroprotective effects in cerebral ischaemia in rats. Neuroreport 9:3955–3959.

Moore CJ, Winder SJ (2012) The inside and out of dystroglycan post-translational modification. Neuromuscul Disord 22:959–965.

Moro S, Gao Z-G, Jacobson KA, Spalluto G (2006) Progress in the pursuit of therapeutic adenosine receptor antagonists. Med Res Rev 26:131–159.

Morris R (1985) Thy-1 in developing nervous tissue. Dev Neurosci 7:133–160.

Moscoso LM, Chu GC, Gautam M, Noakes PG, Merlie JP, Sanes JR (1995) Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/Heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. Dev Biol 172:158–169.

Nagano M, Suzuki H (2003) Quantitative analyses of expression of GDNF and neurotrophins during postnatal development in rat skeletal muscles. Neurosci Res 45:391–399.

Nakajima Y, Kidokoro Y, Klier FG (1980) The development of functional neuromuscular junctions *in vitro*: an ultrastructural and physiological study. Dev Biol 77:52–72.

Nathanson NM (2000) A multiplicity of muscarinic mechanisms: enough signaling pathways to take your breath away. Proc Natl Acad Sci U S A 97:6245–6247.

Nathanson NM (2008) Synthesis, trafficking, and localization of muscarinic acetylcholine receptors. Pharmacol Ther 119:33–43.

Nelson PG, Lanuza MA, Jia M, Li M-X, Tomas J (2003) Phosphorylation reactions in activity-dependent synapse modification at the neuromuscular junction during development. J Neurocytol 32:803–816.

Nelson PG (2005) Activity-dependent synapse modulation and the pathogenesis of Alzheimer disease. Curr Alzheimer Res 2:497–506.

Nguyen QT, Lichtman JW (1996) Mechanism of synapse disassembly at the developing neuromuscular junction. Curr Opin Neurobiol 6:104–112.

Nguyen QT, Parsadanian AS, Snider WD, Lichtman JW (1998) Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science 279:1725–1729.

Nguyen QT, Sanes JR, Lichtman JW (2002) Pre-existing pathways promote precise projection patterns. Nat Neurosci 5:861–867.

Nishizaki T, Sumikawa K (1994) A cAMP-dependent Ca^{2+} signalling pathway at the endplate provided by the gamma to epsilon subunit switch in ACh receptors. Brain Res Mol Brain Res 24:341–345.

Nitkin RM, Smith MA, Magill C, Fallon JR, Yao YM, Wallace BG, McMahan UJ (1987) Identification of agrin, a synaptic organizing protein from Torpedo electric organ. J Cell Biol 105:2471–2478.

Noakes PG, Hornbruch A, Wolpert L (1993) The relationship between migrating neural crest cells and growing limb nerves in the developing chick forelimb. Prog Clin Biol Res 383A:381–390.

Nyström B (1968) Postnatal development of motor nerve terminals in "slow-red" and "fast-white" cat muscles. Acta Neurol Scand 44:363–383.

O'Brien RA, Ostberg AJ, Vrbová G (1978) Observations on the elimination of polyneuronal innervation in developing mammalian skeletal muscle. J Physiol 282:571–582.

Obis T, Besalduch N, Hurtado E, Nadal L, Santafé MM, Garcia N, Tomàs M, Priego M, Lanuza MA, Tomàs J (2015a) The novel protein kinase C epsilon isoform at the adult neuromuscular synapse: location, regulation by synaptic activity-dependent muscle contraction through TrkB signaling and coupling to ACh release. Mol Brain 8:8.

Obis T, Hurtado E, Nadal L, Tomàs M, Priego M, Simon A, Garcia N, Santafé MM, Lanuza MA, Tomàs J (2015b) The novel protein kinase C epsilon isoform modulates acetylcholine release in the rat neuromuscular junction. Mol Brain 8:80.

Ockenga W, Tikkanen R (2015) Revisiting the endocytosis of the M_2 muscarinic acetylcholine receptor. Membranes (Basel) 5:197–213.

Oda K (1984) Age changes of motor innervation and acetylcholine receptor distribution on human skeletal muscle fibres. J Neurol Sci 66:327–338.

Ogata T (1988) Structure of motor endplates in the different fiber types of vertebrate skeletal muscles. Arch Histol Cytol 51:385–424.

Okada K, Inoue A, Okada M, Murata Y, Kakuta S, Jigami T, Kubo S, Shiraishi H, Eguchi K, Motomura M, Akiyama T, Iwakura Y, Higuchi O, Yamanashi Y (2006) The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science 312:1802–1805.

Olah ME, Stiles GL (2000) The role of receptor structure in determining adenosine receptor activity. Pharmacol Ther 85:55–75.

Olianas MC, Onali P, Neff NH, Costa E (1983) Muscarinic receptors modulate dopamine-activated adenylate cyclase of rat striatum. J Neurochem 41:1364–1369.

Oliveira L, Timóteo M, Correia-de-Sá P (2009) Negative crosstalk between M_1 and M_2 muscarinic autoreceptors involves endogenous adenosine activating A_1 receptors at the rat motor endplate. Neurosci Lett 14:127–131.

Oliveira L, Costa AC, Noronha-Matos JB, Silva I, Cavalcante WLG, Timóteo MA, Corrado AP, Dal Belo CA, Ambiel CR, Alves-do-Prado W, Correia-de-Sá P (2015) Amplification of neuromuscular transmission by methylprednisolone involves activation of presynaptic facilitatory adenosine A_{2A} receptors and redistribution of synaptic vesicles. Neuropharmacology 89:64–76.

Ongini E, Fredholm BB (1996) Pharmacology of adenosine A_{2A} receptors. Trends Pharmacol Sci 17:364–372.

Oppenheim RW, Qin-Wei Y, Prevette D, Yan Q (1992) Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 360:755–757.

Padykula HA, Gauthier GF (1970) The ultrastructure of the neuromuscular junctions of mammalian red, white, and intermediate skeletal muscle fibers. J Cell Biol 46:27–41.

Paisansathan C, Xu H, Vetri F, Hernandez M, Pelligrino DA (2010) Interactions between adenosine and K^+ channel-related pathways in the coupling of somatosensory activation and pial arteriolar dilation. Hear Circ Physiol 299:H2009–H2017.

Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT (2011) Synaptic pruning by microglia is necessary for normal brain development. Science 333:1456–1458.

Parnas H, Segel L, Dudel J, Parnas I (2000) Autoreceptors, membrane potential and the regulation of transmitter release. Trends Neurosci 23:60–68.

Patton BL (2003) Basal lamina and the organization of neuromuscular synapses. J Neurocytol 32:883–903.

Peart JN, Headrick JP (2007) Adenosinergic cardioprotection: multiple receptors, multiple pathways. Pharmacol Ther 114:208–221.

Pedata F, Melani A, Pugliese AM, Coppi E, Cipriani S, Traini C (2007) The role of ATP and adenosine in the brain under normoxic and ischemic conditions. Purinergic Signal 3:299–310.

Peng HB, Ali AA, Daggett DF, Rauvala H, Hassell JR, Smalheiser NR (1998) The relationship between perlecan and dystroglycan and its implication in the formation of the neuromuscular junction. Cell Adhes Commun 5:475–489.

Peng HB, Yang J-F, Dai Z, Lee CW, Hung HW, Feng ZH, Ko C-P (2003) Differential effects of neurotrophins and Schwann cell-derived signals on neuronal survival/growth and synaptogenesis. J Neurosci 23:5050–5060.

Pielage J, Fetter RD, Davis GW (2005) Presynaptic spectrin is essential for synapse stabilization. Curr Biol 15:918–928.

Pierce KL, Luttrell LM, Lefkowitz RJ (2001) New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 20:1532–

1539.

Pitts EV, Potluri S, Hess DM, Balice-Gordon RJ (2006) Neurotrophin and Trk-mediated signaling in the neuromuscular system. Int Anesthesiol Clin 44:21–76.

Plomp JJ, Morsch M, Phillips WD, Verschuuren JJ (2015) Electrophysiological analysis of neuromuscular synaptic function in myasthenia gravis patients and animal models. Exp Neurol 270:41–54.

Poo MM and Boulanger LM (1999) Presynaptic depolarization facilitates neurotrophin-induced synaptic potentiation. Nat Neurosci 2:346–351.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2:24–32.

Porrero C, Rubio-Garrido P, Avendaño C, Clascá F (2010) Mapping of fluorescent protein-expressing neurons and axon pathways in adult and developing Thy1-eYFP-H transgenic mice. Brain Res 1345:59–72.

Pousinha PA, Correia AM, Sebastião AM, Ribeiro JA (2010) Predominance of adenosine excitatory over inhibitory effects on transmission at the neuromuscular junction of infant rats. J Pharmacol Exp Ther 332:153–163.

Purves D, Lichtman JW (1980) Elimination of synapses in the developing nervous system. Science 210:153–157.

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492.

Rang H, Dale M, Ritter J, Flower R, Henderson G (2007) Rang and Dale's Pharmacology, 8th ed. Edinburgh: Elsevier. Churchill Livingstone.

Rasband MN, Trimmer JS (2001) Developmental clustering of ion channels at and near the node of Ranvier. Dev Biol 236:5–16.

Reddy LV, Koirala S, Sugiura Y, Herrera AA, Ko CP (2003) Glial cells maintain synaptic structure and function and promote development of the neuromuscular junction *in vivo*. Neuron 40:563–580.

Redfern PA (1970) Neuromuscular transmission in new-born rats. J Physiol 209:701–709.

Reiner C, Nathanson NM (2012) Muscarinic receptor trafficking. Handb Exp Pharmacol 208:61–78.

Reist NE, Werle MJ, McMahan UJ (1992) Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. Neuron 8:865–868.

Ribchester RR, Barry JA (1994) Spatial versus consumptive competition at polyneuronally innervated neuromuscular junctions. Exp Physiol 79:465–494.

Ribchester RR (1988) Activity-dependent and -independent synaptic interactions during reinnervation of partially denervated rat muscle. J Physiol 401:53–75.

Ribeiro JA, Walker J (1975) The effects of adenosine triphosphate and adenosine diphosphate on transmission at the rat and frog neuromuscular junctions. Br J Pharmacol 54:213–218.

Richards DA, Guatimosim C, Rizzoli SO, Betz WJ (2003) Synaptic vesicle pools at the frog neuromuscular junction. Neuron 39:529–541.

Richardson PJ, Kase H, Jenner PG (1997) Adenosine A_{2A} receptor antagonists as new agents for the treatment of Parkinson's disease. Trends Pharmacol Sci 18:338–344.

Riley DA (1977) Spontaneous elimination of nerve terminals from the endplates of developing skeletal myofibers. Brain Res 134:279–285.

Riley DA (1981) Ultrastructural evidence for axon retraction during the spontaneous elimination of polyneuronal innervation of the rat soleus muscle. J Neurocytol 10:425–440.

Rimer M, Mathiesen I, Lømo T, McMahan UJ (1997) Gamma-AChR/epsilon-AChR switch at agrin-induced postsynaptic-like apparatus in skeletal muscle. Mol Cell Neurosci 9:254–263.

Rimer M, Prieto AL, Weber JL, Colasante C, Ponomareva O, Fromm L, Schwab MH, Lai C, Burden SJ (2004) Neuregulin-2 is synthesized by motor neurons and terminal Schwann cells and activates acetylcholine receptor transcription in muscle cells expressing ErbB4. Mol Cell Neurosci 26:271–281.

Ringdahl B, Jenden DJ (1983) Pharmacological properties of oxotremorine and its analogs. Life Sci 32:2401–2413.

Rizo J, Rosenmund C (2008) Synaptic vesicle fusion. Nat Struct Mol Biol 15:665–674.

Rizo J, Xu J (2015) The synaptic vesicle release machinery. Annu Rev Biophys 44:339–367.

Rizzoli SO, Betz WJ (2004) The structural organization of the readily releasable pool of synaptic vesicles. Science 303:2037–2039.

Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. Nat Rev Neurosci 6:57–69.

Robitaille R, Garcia ML, Kaczorowski GJ, Charlton MP (1993) Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. Neuron 11:645–655.

Robitaille R (1995) Purinergic receptors and their activation by endogenous purines at perisynaptic glial cells of the frog neuromuscular junction. J Neurosci 15:7121–7131.

Robitaille R, Bourque MJ, Vandaele S (1996) Localization of L-type Ca^{2+} channels at perisynaptic glial cells of the frog neuromuscular junction. J Neurosci 16:148–158.

Robitaille R, Jahromi BS, Charlton MP (1997) Muscarinic Ca^{2+} responses resistant to muscarinic antagonists at perisynaptic Schwann cells of the frog neuromuscular junction. J Physiol:337–347.

Roche SL, Sherman DL, Dissanayake K, Soucy G, Desmazieres A, Lamont DJ, Peles E, Julien J-P, Wishart TM, Ribchester RR, Brophy PJ, Gillingwater TH (2014) Loss of glial neurofascin155 delays developmental synapse elimination at the neuromuscular junction. J Neurosci 34:12904–12918.

Rochon D, Rousse I, Robitaille R (2001) Synapse-glia interactions at the mammalian neuromuscular junction. J Neurosci 21:3819–3829.

Rotundo RL (2003) Expression and localization of acetylcholinesterase at the

neuromuscular junction. J Neurocytol 32:743–766.

Rotundo RL, Rossi SG, Kimbell LM, Ruiz C, Marrero E (2005) Targeting acetylcholinesterase to the neuromuscular synapse. Chem Biol Interact 157–158:15–21.

Rousse I, St-Amour A, Darabid H, Robitaille R (2010) Synapse-glia interactions are governed by synaptic and intrinsic glial properties. Neuroscience 167:621–632.

Ruegg MA, Tsim KW, Horton SE, Kröger S, Escher G, Gensch EM, McMahan UJ (1992) The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. Neuron 8:691–699.

Salpeter MM, Loring RH (1985) Nicotinic acetylcholine receptors in vertebrate muscle: properties, distribution and neural control. Prog Neurobiol 25:297–325.

Salpeter MM (1987) The vertebrate neuromuscular junction (Alan R Liss I, ed). New York.

Sanes JR, Lichtman JW (1999) Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22:389–442.

Sanes JR, Lichtman JW (2001) Development: Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791–805.

Sanes JR (2003) The basement membrane/basal lamina of skeletal muscle. J Biol Chem 278:12601–12604.

Santafé MM, Garcia N, Lanuza MA, Uchitel OD, Tomàs J (2001) Calcium channels coupled to neurotransmitter release at dually innervated neuromuscular junctions in the newborn rat. Neuroscience 102:697–708.

Santafé MM, Garcia N, Lanuza MA, Uchitel OD, Salon I, Tomàs J (2002) Decreased calcium influx into the neonatal rat motor nerve terminals can recruit additional neuromuscular junctions during the synapse elimination period. Neuroscience 110:147–154.

Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J (2003) Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. Eur J Neurosci 17:119–127.

Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J (2004) Muscarinic autoreceptors related with calcium channels in the strong and weak inputs at polyinnervated developing rat neuromuscular junctions. Neuroscience 123:61–73.

Santafé MM, Lanuza MA, Garcia N, Tomàs J (2005) Calcium inflow-dependent protein kinase C activity is involved in the modulation of transmitter release in the neuromuscular junction of the adult rat. Synapse 57:76–84.

Santafé MM, Lanuza MA, Garcia N, Tomàs J (2006) Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. Eur J Neurosci 23:2048–2056.

Santafé MM, Garcia N, Lanuza MA, Tomàs J (2007a) Protein kinase C activity affects neurotransmitter release at polyinnervated neuromuscular synapses. J Neurosci Res 85:1449–1457.

Santafé MM, Lanuza MA, Garcia N, Tomàs M, Tomàs J (2007b) Coupling of presynaptic muscarinic autoreceptors to serine kinases in low and high release conditions on

the rat motor nerve terminal. Neuroscience 148:432–440.

Santafé MM, Sabaté MM, Garcia N, Ortiz N, Lanuza MA, Tomàs J (2008) Anti-GM2 gangliosides IgM paraprotein induces neuromuscular block without neuromuscular damage. J Neuroimmunol 204:20–28.

Santafé MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Tomàs J (2009a) Presynaptic muscarinic receptors, calcium channels, and protein kinase C modulate the functional disconnection of weak inputs at polyinnervated neonatal neuromuscular synapses. J Neurosci Res 87:1195–1206.

Santafé MM, Garcia N, Lanuza MA, Tomàs M, Tomàs J (2009b) Interaction between protein kinase C and protein kinase A can modulate transmitter release at the rat neuromuscular synapse. J Neurosci Res 87:683–690.

Santafé MM, Garcia N, Tomàs M, Obis T, Lanuza MA, Besalduch N, Tomàs J (2014) The interaction between tropomyosin-related kinase B receptors and serine kinases modulates acetylcholine release in adult neuromuscular junctions. Neurosci Lett 561:171–175.

Santafé MM, Priego M, Obis T, Garcia N, Tomàs M, Lanuza MA, Tomàs J (2015) Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse. Eur J Neurosci 42:1775–1787.

Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA, Stevens B (2012) Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74:691–705.

Schwarz TL (2006) Transmitter release at the neuromuscular junction. In: International review of neurobiology, pp 105–144.

Schwarzschild MA, Agnati L, Fuxe K, Chen J-F, Morelli M (2006) Targeting adenosine A_{2A} receptors in Parkinson's disease. Trends Neurosci 29:647–654.

Searl TJ, Silinsky EM (2005) Modulation of Ca^{2+} -dependent and Ca^{2+} -independent miniature endplate potentials by phorbol ester and adenosine in frog. Br J Pharmacol 145:954–962.

Segal RA (2003) Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosci 26:299–330.

Sendtner M, Schmalbruch H, Stöckli KA, Carroll P, Kreutzberg GW, Thoenen H (1992) Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. Nature 358:502–504.

Shakiryanova D, Tully A, Levitan ES (2006) Activity-dependent synaptic capture of transiting peptidergic vesicles. Nat Neurosci 9:896–900.

Shamim MT, Ukena D, Padgett WL, Daly JW (1989) Effects of 8-phenyl and 8-cycloalkyl substituents on the activity of mono-, di-, and trisubstituted alkylxanthines with substitution at the 1-, 3-, and 7-positions. J Med Chem 32:1231–1237.

Shelton DL, McMahon SB, Winslow JW, Qao WQ (1995) Neurotrophins and neurotrophin antagonists as potential therapeutics. Restor Neurol Neurosci 8:99–100.

Silinsky EM (2004) Adenosine decreases both presynaptic calcium currents and neurotransmitter release at the mouse neuromuscular junction. J Physiol 558:389–

401.

Singhal N, Martin PT (2011) Role of extracellular matrix proteins and their receptors in the development of the vertebrate neuromuscular junction. Dev Neurobiol 71:982–1005.

Slater CR (1982a) Postnatal maturation of nerve-muscle junctions in hindlimb muscles of the mouse. Dev Biol 94:11–22.

Slater CR (1982b) Neural influence on the postnatal changes in acetylcholine receptor distribution at nerve-muscle junctions in the mouse. Dev Biol 94:23–30.

Slater CR (2008) Reliability of neuromuscular transmission and how it is maintained. In: Handbook of clinical neurology, pp 27–101.

Slutsky I, Parnas H, Parnas I (1999) Presynaptic effects of muscarine on ACh release at the frog neuromuscular junction. J Physiol:769–782.

Smith IW, Mikesh M, Lee Y, Thompson WJ (2013) Terminal Schwann cells participate in the competition underlying neuromuscular synapse elimination. J Neurosci 33:17724–17736.

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627–638.

Sofroniew MV, Howe CL, Mobley WC (2001) Nerve growth factor signaling, neuroprotection and neural repair. Annu Rev Neurosci 24:1217–1281.

Spellman DS, Deinhardt K, Darie CC, Chao MV, Neubert TA (2008) Stable isotopic labeling by amino acids in cultured primary neurons: application to brain-derived neurotrophic factor-dependent phosphotyrosine-associated signaling. Mol Cell Proteomics 7:1067–1076.

Steinbach JH (1981) Developmental changes in acetylcholine receptor aggregates at rat skeletal neuromuscular junctions. Dev Biol 84:267–276.

Stocksley MA, Awad SS, Young C, Lightowlers RN, Brenner H-R, Slater CR (2005) Accumulation of NaV1 mRNAs at differentiating postsynaptic sites in rat soleus muscles. Mol Cell Neurosci 28:694–702.

Stoop R, Poo MM (1996) Synaptic modulation by neurotrophic factors. Prog Brain Res 109:359–364.

Südhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375:645–653.

Südhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509–547.

Sugita S, Saito F, Tang J, Satz J, Campbell K, Südhof TC (2001) A stoichiometric complex of neurexins and dystroglycan in brain. J Cell Biol 154:435–445.

Sugiura Y, Woppmann A, Miljanich GP, Ko CP (1995) A novel omega-conopeptide for the presynaptic localization of calcium channels at the mammalian neuromuscular junction. J Neurocytol 24:15–27.

Takamori M (2012) Structure of the neuromuscular junction: function and cooperative mechanisms in the synapse. Ann N Y Acad Sci 1274:14–23.

Talts JF, Andac Z, Göhring W, Brancaccio A, Timpl R (1999) Binding of the G domains of laminin α1 and α2 chains and perlecan to heparin, sulfatides, α-dystroglycan and several extracellular matrix proteins. EMBO J 18:863–870.

Tapia JC, Wylie JD, Kasthuri N, Hayworth KJ, Schalek R, Berger DR, Guatimosim C, Seung HS, Lichtman JW (2012) Pervasive synaptic branch removal in the mammalian neuromuscular system at birth. Neuron 74:816–829.

Thompson WJ (1985) Activity and synapse elimination at the neuromuscular junction. Cell Mol Neurobiol 5:167–182.

Tice MA, Hashemi T, Taylor LA, McQuade RD (1996) Distribution of muscarinic receptor subtypes in rat brain from postnatal to old age. Brain Res Dev Brain Res 92:70–76.

Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR (1979) Laminin--a glycoprotein from basement membranes. J Biol Chem 254:9933–9937.

Timpl R, Brown JC (1996) Supramolecular assembly of basement membranes. BioEssays 18:123–132.

Todd KJ, Robitaille R (2006) Purinergic modulation of synaptic signalling at the neuromuscular junction. Pflügers Arch Eur J Physiol 452:608–614.

Todd KJ, Auld DS, Robitaille R (2007) Neurotrophins modulate neuron-glia interactions at a vertebrate synapse. Eur J Neurosci 25:1287–1296.

Tomàs J, Lanuza MA, Santafé M, Fenoll-Brunet MR, Garcia N (2000) Topological differences along mammalian motor nerve terminals for spontaneous and alpha-bungarotoxin-induced sprouting. Histol Histopathol 15:43–52.

Tomàs J, Santafé MM, Lanuza MA, García N, Besalduch N, Tomàs M (2011) Silent synapses in neuromuscular junction development. J Neurosci Res 89:3–12.

Tomàs J, Santafé MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Obis T, Priego M, Hurtado E (2014) Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. J Neurosci Res 92:543–554.

Trachtenberg JT, Thompson WJ (1996) Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. Nature 379:174–177.

Trinidad JC, Fischbach GD, Cohen JB (2000) The Agrin/MuSK signaling pathway is spatially segregated from the neuregulin/ErbB receptor signaling pathway at the neuromuscular junction. J Neurosci 20:8762–8770.

Turney SG, Lichtman JW (2012) Reversing the outcome of synapse elimination at developing neuromuscular junctions *in vivo*: evidence for synaptic competition and its mechanism Harris WA, ed. PLoS Biol 10:e1001352.

Ukena D, Schudt C, Sybrecht GW (1993) Adenosine receptor-blocking xanthines as inhibitors of phosphodiesterase isozymes. Biochem Pharmacol 45:847–851.

Van Amerongen R, Nusse R (2009) Towards an integrated view of Wnt signaling in development. Development 136:3205–3214.

Van der Kloot W, Molgó J (1994) Quantal acetylcholine release at the vertebrate neuromuscular junction. Physiol Rev 74:899–991.

Vanattou-Saïfoudine N, Gossen A, Harkin A (2011) A role for adenosine A_1 receptor blockade in the ability of caffeine to promote MDMA "Ecstasy"-induced striatal dopamine release. Eur J Pharmacol 650:220–228.

VanDam RJ, Shields EJ, Kelty JD (2008) Rhythm generation by the pre-Bötzinger Complex in medullary slice and island preparations: Effects of adenosine A_1 receptor activation. BMC Neurosci 9:95.

Vidal M, Morris R, Grosveld F, Spanopoulou E (1990) Tissue-specific control elements of the Thy-1 gene. EMBO J 9:833–840.

Villar-Menéndez I, Porta S, Buira SP, Pereira-Veiga T, Díaz-Sánchez S, Albasanz JL, Ferrer I, Martín M, Barrachina M (2014) Increased striatal adenosine A_{2A} receptor levels is an early event in Parkinson's disease-related pathology and it is potentially regulated by miR-34b. Neurobiol Dis 69:206–214.

Walsh MK, Lichtman JW (2003) *In vivo* time-lapse imaging of synaptic takeover associated with naturally occurring synapse elimination. Neuron 37:67–73.

Wang J, Jing Z, Zhang L, Zhou G, Braun J, Yao Y, Wang Z-Z (2003) Regulation of acetylcholine receptor clustering by the tumor suppressor APC. Nat Neurosci 6:1017–1018.

Wang J, Song F, Loeb JA (2017) Neuregulin1 fine-tunes pre-, post-, and peri-synaptic neuromuscular junction development. Dev Dyn.

Wang XH, Poo MM (1997) Potentiation of developing synapses by postsynaptic release of neurotrophin-4. Neuron 19:825–835.

Watson N, Barnes PJ, Maclagan J (1992) Actions of methoctramine, a muscarinic M_2 receptor antagonist, on muscarinic and nicotinic cholinoceptors in guinea-pig airways *in vivo* and *in vitro*. Br J Pharmacol 105:107–112.

Weatherbee SD, Anderson K V., Niswander LA (2006) LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development 133:4993–5000.

Wess J, Angeli P, Melchiorre C, Moser U, Mutschler E, Lambrecht G (1988) Methoctramine selectively blocks cardiac muscarinic M_2 receptors *in vivo*. Naunyn Schmiedebergs Arch Pharmacol 338:246–249.

Wess J (2004) Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. Annu Rev Pharmacol Toxicol 44:423–450.

Wess J, Eglen RM, Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov 6:721–733.

Wessler I, Karl M, Mai M, Diener A (1987) Muscarine receptors on the rat phrenic nerve, evidence for positive and negative muscarinic feedback mechanisms. Naunyn Schmiedebergs Arch Pharmacol 335:605–612.

Wiegert JS, Oertner TG (2013) Long-term depression triggers the selective elimination of weakly integrated synapses. Proc Natl Acad Sci U S A 110:E4510-9.

Wilhelm JC, Xu M, Cucoranu D, Chmielewski S, Holmes T, Lau K, Bassell GJ, English AW (2012) Cooperative roles of BDNF expression in neurons and Schwann cells are modulated by exercise to facilitate nerve regeneration. J Neurosci 32:5002–5009.

Witzemann V (2006) Development of the neuromuscular junction. Cell Tissue Res 326:263–271.

Wolpowitz D, Mason TB, Dietrich P, Mendelsohn M, Talmage DA, Role LW (2000) Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. Neuron 25:79–91.

Woo NH, Lu B (2006) Regulation of cortical interneurons by neurotrophins: from development to cognitive disorders. Neurosci 12:43–56.

Wood SJ, Slater CR (2001) Safety factor at the neuromuscular junction. Prog Neurobiol 64:393–429.

Wright MC, Potluri S, Wang X, Dentcheva E, Gautam D, Tessler A, Wess J, Rich MM, Son Y-J (2009) Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses. J Neurosci 29:14942–14955.

Wu C, Wong T, Wu X, Sheppy E, Zhang L (2009) Adenosine as an endogenous regulating factor of hippocampal sharp waves. Hippocampus 19:205–220.

Wu PH, Phillis JW (1982) Uptake of adenosine by isolated rat brain capillaries. J Neurochem 38:687–690.

Wyatt RM, Balice-Gordon RJ (2003) Activity-dependent elimination of neuromuscular synapses. J Neurocytol 32:777–794.

Xia N-S, Luo W-X, Zhang J, Xie X-Y, Yang H-J, Li S-W, Chen M, Ng M-H (2002) Bioluminescence of Aequorea macrodactyla, a common jellyfish species in the East China Sea. Mar Biotechnol 4:155–162.

Xie ZP, Poo MM (1986) Initial events in the formation of neuromuscular synapse: rapid induction of acetylcholine release from embryonic neuron. Proc Natl Acad Sci U S A 83:7069–7073.

Yang J, Siao C-J, Nagappan G, Marinic T, Jing D, McGrath K, Chen Z-Y, Mark W, Tessarollo L, Lee FS, Lu B, Hempstead BL (2009) Neuronal release of proBDNF. Nat Neurosci 12:113–115.

Yang J, Yang H, Liu Y, Li X, Qin L, Lou H, Duan S, Wang H (2016) Astrocytes contribute to synapse elimination via type 2 inositol 1,4,5-trisphosphate receptor-dependent release of ATP. Elife 5.

Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, Birchmeier C, Burden SJ (2001) Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron 30:399–410.

Ye Y, Rape M (2009) Building ubiquitin chains: E2 enzymes at work. Nat Rev Mol Cell Biol 10:755–764.

Yoshii A, Constantine-Paton M (2010) Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Dev Neurobiol 70:304–322.

Young SH, Poo MM (1983) Spontaneous release of transmitter from growth cones of embryonic neurones. Nature 305:634–637.

Zhai RG, Bellen HJ (2004) The architecture of the active zone in the presynaptic nerve

terminal. Physiology 19:262–270.

Zhang B, Liang C, Bates R, Yin Y, Xiong W-C, Mei L (2012) Wnt proteins regulate acetylcholine receptor clustering in muscle cells. Mol Brain 5:7.

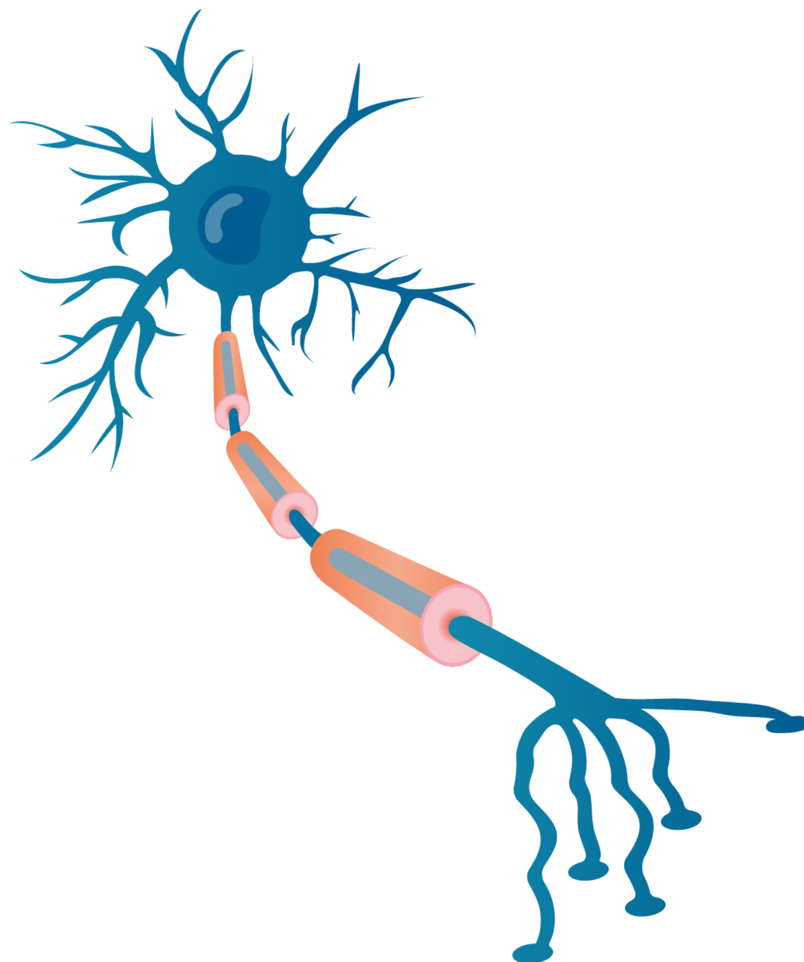
Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L (2008) LRP4 serves as a coreceptor of agrin. Neuron 60:285–297.

Zhang B, Stewart B (2010) Voltage-clamp analysis of synaptic transmission at the Drosophila larval neuromuscular junction. Cold Spring Harb Protoc 2010:pdb.prot5488.

Zhang BG, Quigley AF, Bourke JL, Nowell CJ, Myers DE, Choong PF, Kapsa RM (2016) Combination of agrin and laminin increase acetylcholine receptor clustering and enhance functional neuromuscular junction formation *In vitro*. Dev Neurobiol 76:551–565.

Zini M, Passariello CL, Gottardi D, Cetrullo S, Flamigni F, Pignatti C, Minarini A, Tumiatti V, Milelli A, Melchiorre C, Stefanelli C (2009) Cytotoxicity of methoctramine and methoctramine-related polyamines. Chem Biol Interact 181:409–416.

Zuber B, Unwin N (2013) Structure and superorganization of acetylcholine receptor-rapsyn complexes. Proc Natl Acad Sci 110:10622–10627.



VIII. SCIENTIFIC CONTRIBUTIONS

SCIENTIFIC ARTICLES

Scientific Articles belonging to this Doctoral Thesis:

Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Marta Tomàs, Maria A. Lanuza, Manel M. Santafe, Josep Tomàs. (2016). Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes), adenosine receptors (A_1 and A_{2A}) and tropomyosin-related kinase B receptor (TrkB) modulate in the developmental synapse elimination process at the neuromuscular junction. Molecular Brain 9:67. DOI: 10.1186/s13041-016-0248-9.

Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Marta Tomàs, Maria A. Lanuza, Víctor Cilleros, Josep Tomàs. (2017). Synergistic action of presynaptic muscarinic acetylcholine receptors and adenosine receptors in developmental axonal competition at the neuromuscular junction. Developmental Neuroscience. DOI: 10.1159/000458437.

Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Marta Tomàs, Maria A. Lanuza, Víctor Cilleros, Josep Tomàs. (2017). Presynaptic muscarinic acetylcholine receptors and TrkB receptor cooperate in the elimination of redundant motor nerve terminals during development. Frontiers in Aging Neuroscience 9:24. DOI: 10.3389/fnagi.2017.00024.

Josep Tomàs, Neus Garcia, Maria A. Lanuza, Manel M. Santafé, Marta Tomàs, Laura Nadal, Erica Hurtado, Anna Simó, Victor Cilleros. (2017). Presynaptic membrane receptors modulate ACh release, axonal competition and synapse elimination during neuromuscular junction development. Frontiers in Molecular Neuroscience 10:132. DOI: 10.3389/fnmol.2017.00132.

Josep Tomàs, Neus Garcia, Maria A. Lanuza, Laura Nadal, Marta Tomàs, Erica Hurtado, Anna Simó, Victor Cilleros. Membrane receptor-induced decrease of the protein kinase A and C activity ratio (PKA/PKC ratio) may play a leading role in promoting developmental synapse elimination at the neuromuscular junction. Submitted in Frontiers in Molecular Neuroscience.

Other scientific Articles:

Teresa Obis, Núria Besalduch, Erica Hurtado, Laura Nadal, Manel M. Santafe, Marta Tomàs, Neus Garcia, Mercedes Priego, Maria A. Lanuza, Josep Tomàs. (2015). The novel protein kinase C epsilon isoform at the adult neuromuscular synapse: location, regulation by synaptic activity-dependent muscle contraction through TrkB signaling and coupling to ACh release. Molecular Brain 10;8:8. DOI 10.1186/s13041-015-0098.

Teresa Obis, Erica Hurtado, Laura Nadal, Marta Tomàs, Mercedes Priego, Anna Simón, Neus Garcia, Manel M. Santafe, Maria A. Lanuza, Josep Tomàs. (2015). The novel protein kinase C epsilon isoform modulates acetylcholine release in the rat neuromuscular junction. Molecular Brain 1;8:80. DOI: 10.1186/s13041-015-0171-5.

Erica Hurtado, Víctor Cilleros, Laura Nadal, Anna Simó, Teresa Obis, Neus Garcia, Manel M. Santafe, Marta Tomàs, Katherine Halievski, Cynthia L. Jordan, Maria A. Lanuza, Josep Tomàs. Synaptic activity-induced muscle contraction regulates BDNF/TrkB signaling to retrogradely modulate synaptic function through presynaptic cPKCα and cPKCβI. Accepted in Frontiers in Molecular Neuroscience.

PARTICIPATION IN NATIONAL AND INTERNATIONAL CONGRESSES

Congress: 9th FENS Forum of Neuroscience.
Date and Place: 5-9 July 2014; Milan (Italy).
Authors: Laura Nadal, Erica Hurtado, Teresa Obis, Neus Garcia, Mercedes Priego, Manel M. Santafé, Marta Tomàs, Nicolàs Ortiz, Maria Angel Lanuza and Josep Tomàs.
Title: Blockade of tyrosine kinase receptor B prevents muscle contraction-induced presynaptic nPKCε, cPKCβI and cPKCα increases.
Format: Poster.

Congress: 9th FENS Forum of Neuroscience.
Date and Place: 5-9 July 2014; Milan (Italy).
Authors: Erica Hurtado, Teresa Obis, Mercedes Priego, Laura Nadal, Neus Garcia, Manel M. Santafé, Marta Tomàs, Nicolàs Ortiz, Maria Angel Lanuza and Josep Tomàs.
Title: Colocalization of protein kinase A (PKA) subunits and the A kinase anchoring proteins (AKAPS 9 and 150) in the neuromuscular synapse.
Format: Poster.

Congress: 9th FENS Forum of Neuroscience.
Date and Place: 5-9 July 2014; Milan (Italy).
Authors: Teresa Obis, Núria Besalduch, Manel M. Santafé, Marta Tomàs, Neus Garcia, Mercedes Priego, Erica Hurtado, Laura Nadal, Maria Angel Lanuza and Josep Tomàs.
Title: Activity-dependent changes of the novel protein kinase C epsilon isoform in the neuromuscular synapse and its coupling to ACh release.
Format: Poster.

Congress: 9th FENS Forum of Neuroscience.
Date and Place: 5-9 July 2014; Milan (Italy).
Authors: Manel M. Santafé, Mercedes Priego, Teresa Obis, Neus Garcia, Marta Tomàs, Maria Angel Lanuza, Nicolàs Ortiz, Erica Hurtado, Laura Nadal and Josep Tomàs.
Title: Purinergic adenosine receptors and cholinergic muscarinic receptors cooperate in acetylcholine release modulation on neuromuscular synapse.
Format: Poster.

Congress: IX Symposium of Neurobiology.
Date and Place: 22-23 October 2014; Barcelona (Spain).
Authors: Laura Nadal, Erica Hurtado, Teresa Obis, Neus Garcia, Manel Santafé, Marta Tomàs, Mercedes Priego, Maria Angel Lanuza, Josep Tomàs.
Title: Activity-dependent changes of the nPKCε isoform through TrkB function in the adult rat neuromuscular synapse.
Format: Oral communication (Laura Nadal)

Congress: IX Symposium of Neurobiology.

Date and Place: 22-23 October 2014; Barcelona (Spain).

Authors: Erica Hurtado, Laura Nadal, Teresa Obis, Neus Garcia, Mercedes Priego, Manel Santafé, Marta Tomàs, Nicolàs Ortiz, Maria Angel Lanuza, Josep Tomàs.

Title: Blockade of tyrosine kinase receptor B prevents muscle contraction-induced presynaptic nPKCε, cPKCβI and cPKCα increases.

Format: Poster.

Congress: 44th Annual Meeting of Society for Neuroscience (SfN 2014).

Date and Place: 15-19 November 2014; Washington (USA).

Authors: Maria Angel Lanuza, Erica Hurtado, Nuria Besalduch, Teresa Obis, Laura Nadal, Neus Garcia, Manel M. Santafé, Mercedes Priego, Marta Tomàs, Josep Tomàs.

Title: The novel protein kinase C epsilon isoform at the neuromuscular synapse: location, synaptic activity-related expression, phosphorylation function and coupling to ACh release.

Format: Poster.

Congress: 44th Annual Meeting of Society for Neuroscience (SfN 2014).

Date and Place: 15-19 November 2014; Washington (USA).

Authors: Neus Garcia, Mercedes Priego, Manel M. Santafé, Teresa Obis, Marta Tomàs, Maria Angel Lanuza, Nicolau Ortiz, Erica Hurtado, Laura Nadal, Josep Tomàs.

Title: Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation on neuromuscular synapse.

Format: Poster.

Congress: 16th National Congress of the Spanish Society for Neuroscience (SENC).

Date and Place: 23-25 September 2015; Granada (Spain).

Authors: Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Marta Tomàs, Maria Angel Lanuza, Manel M. Santafé, and Josep Tomàs.

Title: Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes) modulate the developmental synapse elimination process on the neuromuscular junction.

Format: Poster.

Congress: 16th National Congress of the Spanish Society for Neuroscience (SENC).
Date and Place: 23-25 September 2015; Granada (Spain).
Authors: Erica Hurtado, Laura Nadal, Teresa Obis, Anna Simó, Víctor Cilleros, Neus Garcia, Manel M. Santafé, Marta Tomàs, Maria Angel Lanuza, and Josep Tomàs.
Title: BDNF and TrkB are regulated by both pre- and postsynaptic activity and enhance presynaptic cPKCβI to modulate neuromuscular synaptic function.
Format: Poster.

Congress: 16th National Congress of the Spanish Society for Neuroscience (SENC).
Date and Place: 23-25 September 2015; Granada (Spain).
Authors: Anna Simó, Erica Hurtado, Laura Nadal, Víctor Cilleros, Neus Garcia, Manel M. Santafé, Marta Tomàs, Maria Angel Lanuza, and Josep Tomàs.
Title: Synaptic activity and PKC-TrkB signaling modulates the phosphorylation of the exocytotic proteins SNAP-25 and Munc18-1 at adult neuromuscular junction.
Format: Poster.

Congress: 45th Annual Meeting of Society for Neuroscience (SfN 2015).
Date and Place: 17-21 October 2015; Chicago (USA).
Authors: Maria Angel Lanuza, Erica Hurtado, Laura Nadal, Teresa Obis, Anna Simó, Víctor Cilleros, Neus Garcia, Manel M. Santafé, Marta Tomàs, Josep Tomàs.
Title: BDNF and TrkB are regulated by both pre- and postsynaptic activity and enhance presynaptic cPKCβI to modulate neuromuscular synaptic function.
Format: Poster.

Congress: 10th FENS Forum of Neuroscience.
Date and Place: 2-6 July 2016; Copenhagen (Denmark).
Authors: Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Víctor Cilleros, Marta Tomàs, Maria Angel Lanuza, Manel M. Santafé, and Josep Tomàs.
Title: Muscarinic acetylcholine autoreceptors, adenosine receptors and tropomyosin-related kinase B receptor (TrkB) cooperate in the developmental axonal loss and synapse elimination process at the neuromuscular junction.
Format: Poster.

Congress: 10th FENS Forum of Neuroscience.
Date and Place: 2-6 July 2016; Copenhagen (Denmark).

Authors: Erica Hurtado, Víctor Cilleros, Laura Nadal, Teresa Obis, Anna Simó, Neus Garcia, Manel M. Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.
Title: Synaptic activity-modulated BDNF/TrkB pathway enhances presynaptic cPKCβI to control neuromuscular synaptic function.
Format: Poster.

Congress: 10th FENS Forum of Neuroscience.
Date and Place: 2-6 July 2016; Copenhagen (Denmark).
Authors: Anna Simó, Erica Hurtado, Víctor Cilleros, Laura Nadal, Neus Garcia, Manel M. Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.
Title: BDNF-TrkB/PKC signaling modulated by synaptic activity controls the phosphorylation of the exocytotic proteins Munc18-1 and SNAP25 at the neuromuscular junction.
Format: Poster.

Congress: X Symposium of Neurobiology.
Date and Place: 6-7 October 2016; Barcelona (Spain).
Authors: Laura Nadal, Neus Garcia, Erica Hurtado, Anna Simó, Víctor Cilleros, Laia Just, Marta Tomàs, Maria Angel Lanuza, Manel M. Santafé, and Josep Tomàs.
Title: Adenosine receptors, mAChRs and TrkB modulate the developmental synapse elimination process at the neuromuscular junction.
Format: Poster.

Congress: X Symposium of Neurobiology.
Date and Place: 6-7 October 2016; Barcelona (Spain).
Authors: Erica Hurtado, Víctor Cilleros, Laura Nadal, Teresa Obis, Anna Simó, Neus Garcia, Manel M. Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.
Title: Synaptic activity-modulated BDNF/TrkB pathway enhances presynaptic cPKCβI to control neuromuscular synaptic function.
Format: Poster.

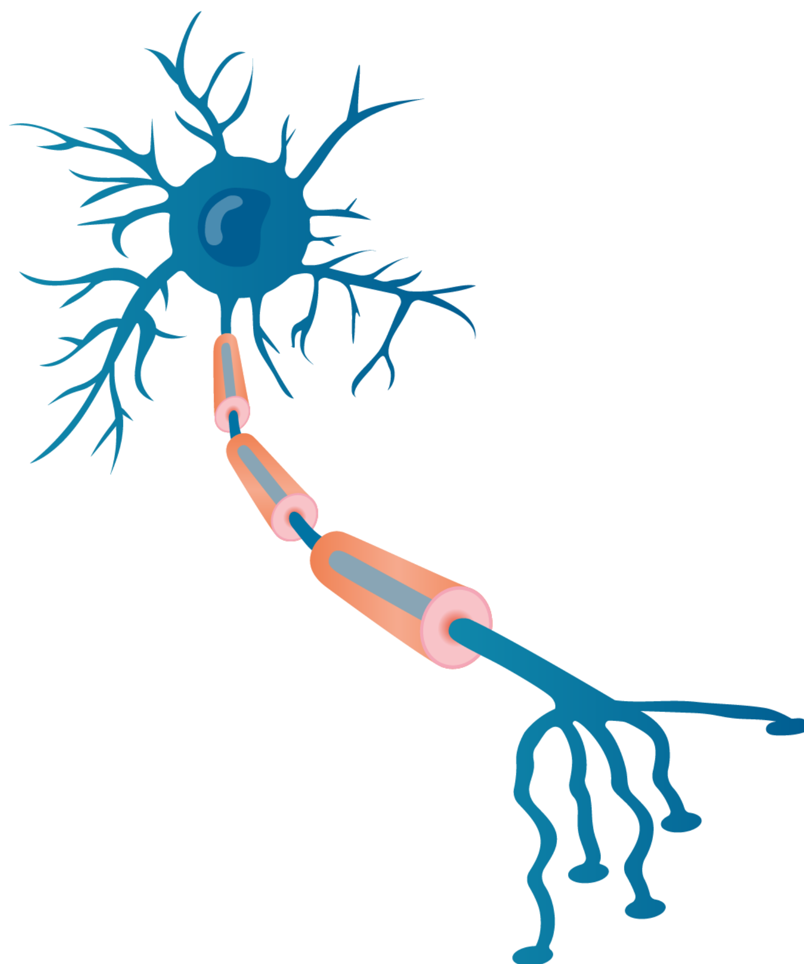
Congress: X Symposium of Neurobiology.
Date and Place: 6-7 October 2016; Barcelona (Spain).
Authors: Anna Simó, Erica Hurtado, Víctor Cilleros, Laura Nadal, Neus Garcia, Manel M. Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.

Title: BDNF-TrkB-PKC signaling modulated by synaptic activity controls the phosphorylation of the exocytotic proteins Munc18-1 and SNAP25 at the neuromuscular junction.
Format: Poster.

Congress: X Symposium of Neurobiology.
Date and Place: 6-7 October 2016; Barcelona (Spain).
Authors: Víctor Cilleros, Erica Hurtado, Anna Simó, Laura Nadal, Laia Just, Teresa Obis, Manel M. Santafé, Marta Tomàs, Neus Garcia, Maria Angel Lanuza and Josep Tomàs.
Title: Neuromuscular activity modulates the signaling of the M_2 subtype muscarinic cholinergic receptor on PKC and on SNAP25 and Munc18-1 phosphorylation.
Format: Oral communication (Víctor Cilleros)

MOBILITY

Institution: Department of Neurological and Movement Sciences in Section of Physiology and Psychology (University of Verona, Italy).
Supervisors: Dr. Giuseppe Busetto.
Objective: Homeostatic role of postsynaptic action potential firing activity on synaptic plasticity.
Length: 3 months (January-April 2016).



APPENDIX I

UNIVERSITAT ROVIRA I VIRGILI
Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process
Laura Nadal Magriñà

Nadal *et al. Molecular Brain* (2016) 9:67
DOI 10.1186/s13041-016-0248-9

Molecular Brain

RESEARCH Open Access

Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes), adenosine receptors (A_1 and A_{2A}) and tropomyosin-related kinase B receptor (TrkB) modulate the developmental synapse elimination process at the neuromuscular junction

Laura Nadal, Neus Garcia[*†], Erica Hurtado, Anna Simó, Marta Tomàs, Maria A. Lanuza[†], Manel Santafé and Josep Tomàs[*†]

Abstract

Background: The development of the nervous system involves an initially exuberant production of neurons that make an excessive number of synaptic contacts. The initial overproduction of synapses promotes connectivity. Hebbian competition between axons with different activities (the least active are punished) leads to the loss of roughly half of the overproduced elements and this refines connectivity and increases specificity. The neuromuscular junction is innervated by a single axon at the end of the synapse elimination process and, because of its relative simplicity, has long been used as a model for studying the general principles of synapse development. The involvement of the presynaptic muscarinic ACh autoreceptors may allow for the direct competitive interaction between nerve endings through differential activity-dependent acetylcholine release in the synaptic cleft. Then, the most active ending may directly punish the less active ones. Our previous results indicate the existence in the weakest axons on the polyinnervated neonatal NMJ of an ACh release inhibition mechanism based on mAChR coupled to protein kinase C and voltage-dependent calcium channels. We suggest that this mechanism plays a role in the elimination of redundant neonatal synapses.

Results: Here we used confocal microscopy and quantitative morphological analysis to count the number of brightly fluorescent axons per endplate in P7, P9 and P15 transgenic B6.Cg-Tg (Thy1-YFP)16 Jrs/J mice. We investigate the involvement of individual mAChR M_1-, M_2- and M_4-subtypes in the control of axonal elimination after the *Levator auris longus* muscle had been exposed to agonist and antagonist *in vivo*. We also analysed the role of adenosine receptor subtypes (A_1 and A_{2A}) and the tropomyosin-related kinase B receptor. The data show that postnatal axonal elimination is a regulated multireceptor mechanism that guaranteed the monoinnervation of the neuromuscular synapses.

(Continued on next page)

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Conclusion: The three receptor sets considered (mAChR, AR and TrkB receptors) intervene in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the lossers but, thereafter, the final elimination would occur with some autonomy and independently of postsynaptic maturation.

Keywords: Motor end-plate, Motor nerve terminal, Cholinergic synapses, Neuromuscular junction

Background

The development of the nervous system involves the initial overproduction of synapses, which promotes connectivity, and a subsequent activity-dependent reduction in the number of synapses. This refines connectivity and increases specificity. Hebbian competition between axons with different activities (the least active are eliminated) seems to be a characteristic of the process and leads to the loss of roughly half of the overproduced elements and the functional consolidation of the remaining synapses in the adult [1, 2]. Synaptic contacts are lost throughout the nervous system during histogenesis [3, 4]. In newborn animals, the skeletal muscle fibers are polyinnervated by several motor axons [5] but at the end of the axonal competition, the endplates are innervated by a single axon. Because of its relative simplicity, the neuromuscular junction (NMJ) has long been used as a model for studying the general principles of synapse development in an attempt to understand the synapse elimination process [2, 6–12].

Various presynaptic receptors seem to play an important role in the axonal competition leading to synapse loss in the NMJ. The involvement of muscarinic acetylcholine autoreceptors (mAChRs) in the elimination process may allow direct competitive interaction between nerve endings through a differential activity-dependent acetylcholine (ACh) release. Then, the more active ending may directly punish those that are less active or reward themselves if the suitable mAChR subtypes are present in the competing axons. Our previous results indicate that, in postnatal muscles, there is an ACh release inhibition mechanism based on mAChR coupled to a PKC-VDCC intracellular cascade. In certain weak motor axons, this mechanism can depress ACh release and even disconnect synapses [13–17]. We suggest that this mechanism plays a central role in the elimination of redundant neonatal synapses because functional axonal withdrawal can indeed be reversed by mAChR, protein kinase C (PKC) or voltage-dependent calcium channels (VDCC) block [17, 18]. However, local differential effectiveness and differential activity will determine eventual success, since an axon that fails at one synapse (muscle cell) may be successful at another [19], which suggests complex regulation involving other receptors and postsynaptic- (and glial cell) derived factors. Both neurotrophin receptors (NTR) and adenosine receptors (AR) belong to leading presynaptic signalling pathways. In the adult NMJ, the activity of one of these receptors can modulate a given combination of spontaneous, evoked and activity-dependent release conditions and a close dependence between them exist [20]. These receptors and their intracellular signalling may help to refine the molecular and structural organization of the newborn synapses so that they can acquire their mature form.

Here we investigate the involvement of individual mAChR subtypes in the control of synapse elimination. We also analyse the role of AR (A_1 and A_{2A}) and trompomyosin-related kinase B receptor (TrkB). The data show that the three receptor sets considered cooperate in the elimination process and promote axonal disconnection at the beginning of the second postnatal week independently of the postsynaptic maturation of the nicotinic receptor cluster.

Results

Postnatal elimination of nerve terminals

Normal evolution of postnatal polyneuronal innervation in the NMJ

Figure 1 shows some representative confocal immunofluorescence images of singly- and polyinnervated NMJs from YFP (autofluorescent axons) and C57BL/J6 (axons stained with anti neurofilament fluorescent antibody) mice. The images show that it is feasible to accurately count the number of axons in both preparations. Firstly, we investigated in our experimental model the normal postnatal elimination of the excess synaptic contacts. Figure 2a shows axonal counts in fluorescent immunohistochemistry LAL preparations (average number of axonal connections per NMJ) from B6.Cg-Tg (Thy1-YFP) – hereafter YFP – and C57BL/J6 mice. The figure also shows previous data [10, 21–23] from Sprague-Dawley (SD) rats to show similarities between rodents. The histogram in Fig. 2b shows the percentage of singly-, dually- and triply- (or more) innervated synapses in YFP for the postnatal days (P) considered without any experimental manipulation (control, non-PBS). Because the present work is based in subcutaneous injection procedure, we also wanted to control that subcutaneous injection by

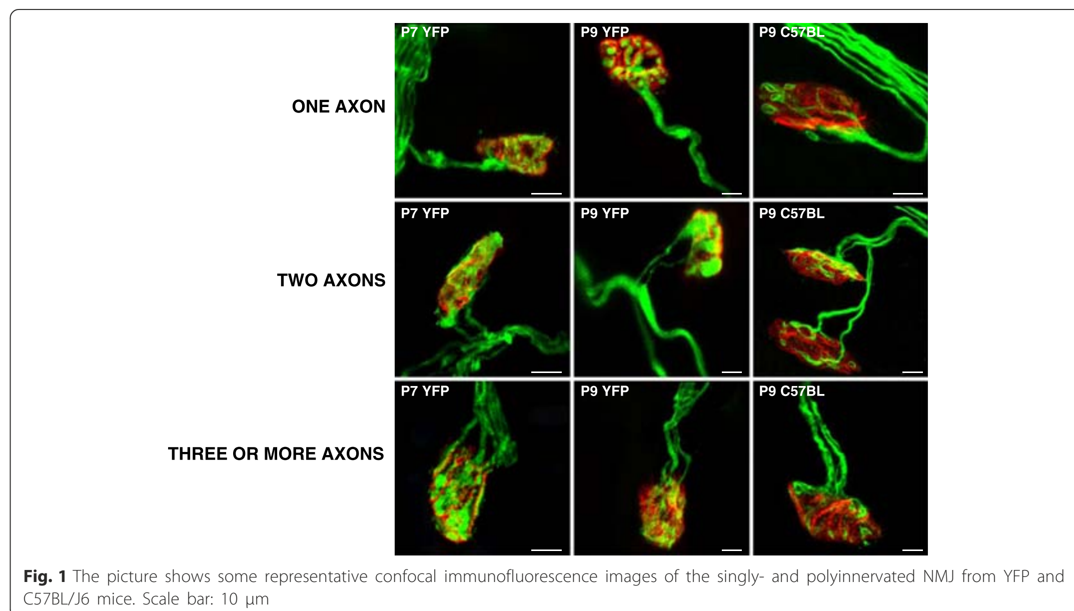

Fig. 1 The picture shows some representative confocal immunofluorescence images of the singly- and polyinnervated NMJ from YFP and C57BL/J6 mice. Scale bar: 10 μm

itself does not affect the elimination process in the control animals. Figure 2b also shows the percentage of singly-, dually- and triply- (or more) innervated synapses at P7 ($n = 2315$ NMJs, $N = 10$ mice), P9 ($n = 2647$ NMJs, $N = 10$ mice) and P15 ($n = 1056$ NMJs, $N = 4$ mice) after two (days 5, 6), four (days 5–8) and ten (days 5–14) subcutaneous PBS injections, respectively (control PBS). No significant differences are observed between PBS (the control for subcutaneous injections) and non-PBS (without subcutaneous injection) preparations ($p >$ 0,05, Fisher's test).

Stimulation of the mAChR. Effect of oxotremorine

Figure 3 shows the percentage of singly-, doubly- and triply (or more) innervated NMJs in the untreated YFP control mice and after 2 (P7), 4 (P9) and in some cases 10 (P15) applications (one application every day after P5) of the mAChR agonist oxotremorine (OXO) and such antagonists as atropine (AT), pirenzepine (PIR), methoctramine (MET) and muscarinic toxin 3 (MT3). We first used the potent and well characterized unselective agonist OXO. A subcutaneous application on the YFP LAL muscle surface every day (at P5 and P6) results in a significant acceleration at P7 of the axonal elimination process (Fig. 3a; Fisher's test; $n = 820$ NMJs, $N = 4$ mice), because of the increase in monoinnervated NMJs ($p < 0{,}005$) and the reduction in dual ($p < 0{,}05$) synapses. It seems that the muscarinic mechanism, when stimulated, accelerates the axonal elimination rate and transition to the monoinnervation state.

However, four applications (P5-P8) of OXO (Fig. 3b; Fisher's test; $n = 865$ NMJs, $N = 4$ mice) do not lead to any significant change at P9 ($p > 0{,}05$) in monoinnervated and dual junctions though a small increase of the fraction of synapses with three or more axons is observed ($p < 0{,}05$). This indicates that the effect of muscarinic stimulation diminishes and tends to peak close to the normal values of axonal elimination around four days after stimulation has begun. Therefore, there is a window around P5-P6 in which mAChR can be forced to accelerate synapse elimination. However, exogenous stimulation with the agonist reveals only that muscarinic signalling has the potential to accelerate postnatal axonal disconnection but does not explain what the tonic muscarinic control is like in a normal situation. Therefore, we investigate how blocking the M_1, M_2 and M_4 mAChR subtypes *in toto* or selectively (those subtypes observed in functional developing NMJ, [13, 14, 24, 25]) can affect synapse elimination.

Unselective inhibition of mAChRs. Effect of atropine

Figure 3a shows that two subcutaneous applications of AT (at P5 and P6) in the YFP LAL muscles analysed at P7 significantly reduce the percentage of triple junctions ($p < 0{,}05$), increase the percentage of dual junctions ($p < 0{,}01$), and have no effect on the percentage of single junctions (Fisher's test; $n = 1343$ NMJs, $N = 3$ mice). Thus, the rate of transition from three to two speeds up but the overall process does not continue to the point of significantly increasing

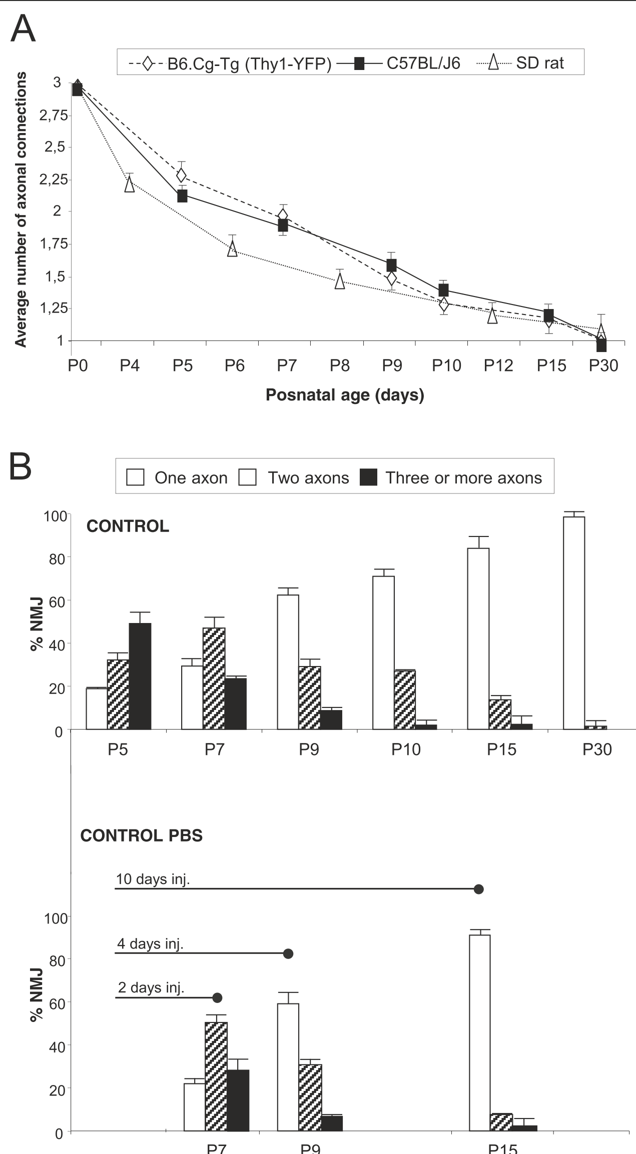

Fig. 2 Postnatal evolution of polyneuronal innervation. In **a**, comparison of the results of axon counts in fluorencence immunohistochemistry LAL preparations of YFP and C57BL/J6 mice. The histogram in **b** shows the percentage of singly-, dually- and triply- (or more) innervated synapses in YFP animals on the postnatal days studied without any experimental manipulation (control non-PBS, without subcutaneous injection), and also at P7, P9 and P15 after two (days 5–6), four (days 5–8) and ten (days 5–14) daily subcutaneous PBS applications respectively (control PBS). No differences are observed between PBS and non-PBS preparations (Fisher's test: $p > 0,05$)

monoinnervation. This indicates that AT has a dual effect: namely, it increases axon loss in triple junctions and reduces loss in double NMJs. It seems that NMJs or nerve terminals of different levels of maturity have different sensitivities and respond differently to this potent muscarinic pan-inhibitor.

Daily AT applications between P5 and P8 lead to a significant retardation of axonal elimination at P9 (Fig. 3b;

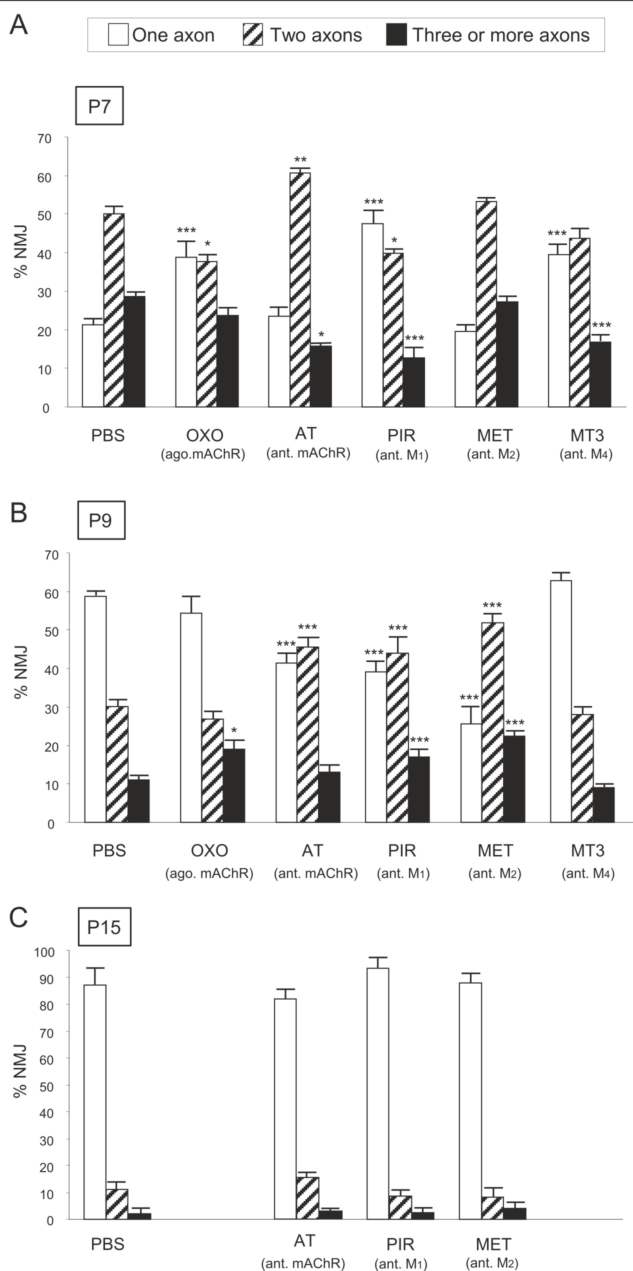

Fig. 3 Changes in polyneuronal innervation of the NMJ after stimulation and inhibition of the mAChR. The figure shows the percentage of singly-, dually- and triply- (or more) innervated NMJs in the untreated YFP control mice (exposed to PBS applications) and after 2 (P7 in **a**), 4 (P9, in **b**) and in some cases 10 (P15, in **c**) applications (one application every day after P5) of the mAChR agonist (ago.) oxotremorine (OXO) and such antagonists (ant.) as atropine (AT), pirenzepine (PIR), methoctramine (MET) and muscarinic toxin 3 (MT3). Fisher's test: * $p < 0{,}05$, ** $p < 0{,}01$, *** $p < 0{,}005$

Fisher's test; $n = 1032$ NMJs, $N = 4$ mice) with persistent polyinnervation due to the higher percentage of dual junctions ($p < 0{,}005$) the corresponding decrease in monoinnervated synapses ($p < 0{,}005$) and an almost normal number of triple junctions ($p > 0{,}05$). This clearly indicates that blocking the mAChR can persistently obstruct the two-to-one transition of the elimination processs. However, unlike the OXO effect (which tends to disappear at P9 after accelerating elimination at P7), the effect of AT seems to be maintained throughout the period P5-P9 at least in relation to the two-to-one transition. It seems, then, that in normal conditions, the presynaptic muscarinic mechanism increases the rate of axonal loss at least in dual junctions in the period P5-P9 and that this effect can be increased at P7 by using an exogenous agonist.

Selective block of the mAChRs

How are the various mAChR subtypes that operate in the postnatal NMJ (M_1, M_2 and M_4; [16]) involved in axonal elimination? We selectively blocked M_1 (PIR), M_2 (MET) and M_4 (MT3) and observed the NMJ at P7 (daily applications on the LAL surface at P5 and P6, Fig. 3a) and P9 (applications between P5-P8, Fig. 3b). At P7 two subcutaneous PIR applications significantly reduced the percentage of triple ($p < 0{,}005$) and dual junctions ($p < 0{,}05$) and greatly increased the percentage of single junctions ($p < 0{,}005$, Fisher's test; $n = 915$ NMJs, $N = 4$ mice). Thus, both the three-to-two and the two-to-one rates of transition accelerated considerably and the overall elimination process speeded up. This may indicate that in the normal situation the role of M_1 is to slow elimination down because when it is uncoupled from PIR, the elimination process accelerates. Interestingly, the M_4 blocker MT3 has almost exactly the same effect as the M_1 blocker PIR (Fisher's test; $n = 895$ NMJs, $N = 4$ mice), whereas the M_2 blocker MET does not have a significant effect at P7 after the two subcutaneous applications ($p > 0{,}05$, Fisher's test; $n = 1012$ NMJs, $N = 4$ mice). As an additional control, in P7 C57BL/J6 animals treated with MET we found the same result (Fisher's test; Control PBS ($n = 1533$ NMJs, $N = 6$ mice): 1 axon: 22.69 % ± 1,04 % ; 2 axons: 50.20 % ± 2.75 % ; 3 or more axons: 27.11 % ± 3.18 %. MET application ($n = 911$ NMJs, $N = 3$ mice): 1 axon: 22.22 % ± 2.56 % ($p > 0{,}05$); 2 axons: 50.00 % ± 2.74 % ($p > 0{,}05$); 3 or more axons: 27.78 % ± 2.38 ($p > 0{,}05$)). Thus, at P7 the ensemble M_1/M_4 seems to be involved in a mechanism that delays elimination because when it is blocked the percentage of monoinnervated junctions increased and caused a fast three-to-one transition.

Nevertheless, how can it be explained that at this time (P7) the two-to-one transition is accelerated by the selective blockers PIR and MT3 (and not affected by MET), but that when all mAChR subtypes were blocked with AT this transition was partially delayed? Blocking the whole ensemble of subtypes with AT has a somehow diferent effect than the individual effects of mAChR subtypes. This apparent contradiction observed with the effects of selective and unselective pharmacological muscarinic inhibitory substances at P7 seems to suggest the existence of other confluent signalling pathways that take part in the process (see below).

However, daily applications of these substances for four days (P5-P8) lead to a much more clearly defined situation at P9 (Fig. 3b). As stated above, four AT applications delay elimination, maintain the number of dual junctions and decrease the number of singly-innervated NMJ, which indicates that the two-to-one transition is slowing down. The same effect (even greater because of the considerable delay in the three-to-two transition) is obtained by blocking M_1 (PIR, $p < 0{,}005$, Fisher's test; $n = 1293$ NMJs, $N = 3$ mice) and M_2 (MET, $p < 0{,}005$, $n = 976$ NMJs, $N = 4$ mice) but not in this case with the M_4 blocker MT3 ($p > 0{,}05$, $n = 1177$ NMJs, $N = 4$ mice). As an additional control, in P9 C57BL/J6 animals treated with MT3 we found the same result (Fisher's test; Control PBS ($n = 1352$ NMJs, $N = 5$ mice): 1 axon: 48.17 % ± 4.54 % ; 2 axons: 36.73 % ± 2.76 % ; 3 or more axons: 15.10 % ± 4.97 %.MT3 aplications ($n = 906$ NMJs, $N = 4$ mice): 1 axon: 51.2 % ± 5.77 % ($p > 0.05$); 2 axons: 39.32 % ± 2.53 % ($p > 0.05$); 3 or more axons: 9.48 % ± 2.32 ($p > 0.05$)). These data indicate that at this point in the elimination process, both M_1 and M_2 subtypes cooperate in favouring the full sequence of synapse elimination.

To investigate the possible persistence of the mAChR effect throughout the period of synapse elimination, we made daily applications of AT (the unselective mAChR antagonist), PIR and MET (the M_1 and M_2 selective antagonists that are effective at modulating axonal elimination at P9) between P5 and P15 (in normal conditions almost 90% of NMJs were monoinnervated at P15). In spite of the continued presence of unselective and selective inhibitors, we found that the elimination process came to its normal conclusion by the end of the second postnatal week (Fig. 3c; ($p > 0{,}05$, Fisher's test; AT: $n = 720$ NMJs, $N = 3$ mice; PIR: $n = 924$ NMJs, $N = 3$ mice; MET: $n = 870$ NMJs, $N = 3$ mice)). This reinforces the suggestion that several signalling mechanisms between the endings in competition cooperate (and substitute each other) to resolve the correct synaptic connection in a multifactorial process.

Other signalling mechanisms involved in axonal loss

Several signalling pathways connect the cells that make synapses. Here, we studied the possible involvement of adenosine receptors and neurotrophin receptors (here the representative TrkB receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) in the

complex period of axonal elimination around P7-P9 (Fig. 4). To the LAL muscle, we subcutaneously applied the AR inhibitor 8SPT, the AR agonist ADO and the TrkB blocking pathway agent TrkB-Fc to sequester endogenous BDNF/NT-4 neurotrophins. With the 8SPT ($n = 920$ NMJs, $N = 4$ mice) and TrkB-Fc ($n = 1113$ NMJs, $N = 4$

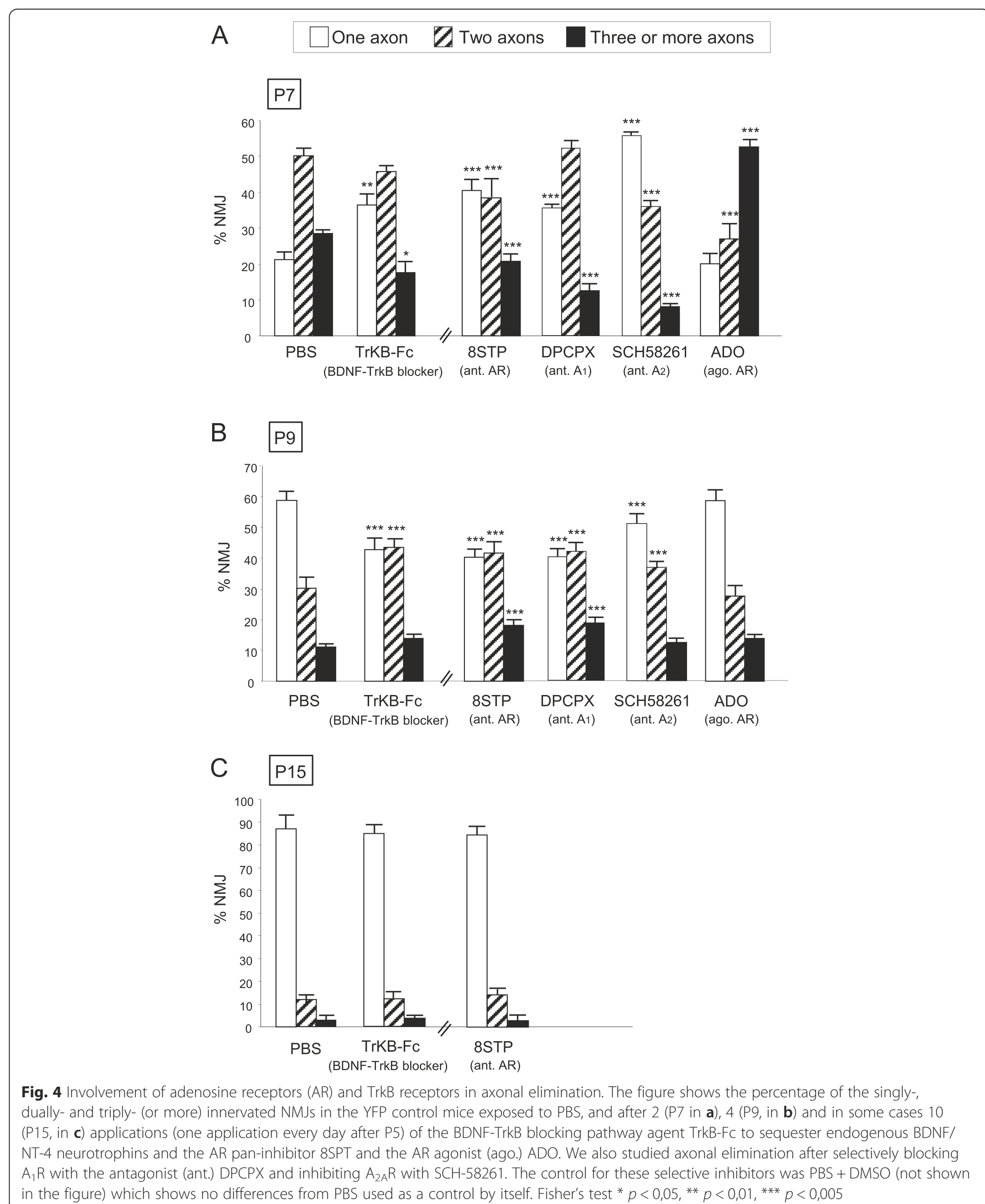

Fig. 4 Involvement of adenosine receptors (AR) and TrkB receptors in axonal elimination. The figure shows the percentage of the singly-, dually- and triply- (or more) innervated NMJs in the YFP control mice exposed to PBS, and after 2 (P7 in **a**), 4 (P9, in **b**) and in some cases 10 (P15, in **c**) applications (one application every day after P5) of the BDNF-TrkB blocking pathway agent TrkB-Fc to sequester endogenous BDNF/NT-4 neurotrophins and the AR pan-inhibitor 8SPT and the AR agonist (ago.) ADO. We also studied axonal elimination after selectively blocking A_1R with the antagonist (ant.) DPCPX and inhibiting $A_{2A}R$ with SCH-58261. The control for these selective inhibitors was PBS + DMSO (not shown in the figure) which shows no differences from PBS used as a control by itself. Fisher's test * $p < 0,05$, ** $p < 0,01$, *** $p < 0,005$

mice) blockers at P7 we observed a clear acceleration in the three-to-two rate (Fisher's test; 8SPT: $p < 0{,}005$; TrkB-Fc: $p < 0{,}05$) that was very similar to the acceleration in the two-to-one rate. These substances accelerate axonal elimination on the NMJ and, therefore, the physiological role in normal conditions of the AR and TrkB pathways at P7 seems to delay the axonal loss process. This is confirmed for the AR because exposure to the physiological agonist ADO results in a significantly higher number of triple junctions and a significant reduction in the number of dual junctions ($p < 0{,}005$, Fisher's test; $n = 923$ NMJs, $N = 4$ mice). This indicates an ADO-induced retardation of axonal elimination. Which AR subtypes are involved in the ADO effect? We analysed axonal elimination after selectively blocking A_1R with DPCPX ($n = 1160$ NMJs, $N = 4$ mice) or $A_{2A}R$ inhibition with SCH-58261($n = 963$ NMJs, $N = 4$ mice) (Fig. 4a). The data show that axonal loss (the full three-to-one transition) is accelerated by both inhibitors ($p < 0{,}005$), which indicates that in normal conditions without inhibition both A_1R and $A_{2A}R$ are associated with delaying loss.

Interestingly, at P9, neurotrophin signalling seems to reverse their coupling to the axonal loss process because TrkB-Fc (acting between P5-P8) considerably delays elimination (resulting in more dual and fewer monoinnervated NMJ; $p < 0{,}005$, Fisher's test; $n = 863$ NMJs, $N = 4$ mice), which indicates that in a normal situation BDNF/NT-4 mediators change their role and accelerate elimination, as has been described above for the muscarinic mechanism. At P9, the purinergic mechanism also seems to tonically accelerate axonal elimination to the maximum rate because the AR pan-inhibitor 8SPT delays the process (an effect of the A_1R and $A_{2A}R$, Fig. 4b; Fisher's test; $n > 900$ NMJs, $N = 4$ mice in each case) with no effect of the agonist ADO ($p > 0{,}05$, $n = 908$ NMJs, $N = 4$ mice).

Therefore, it seems that AR may behave biphasically in the critical period between 5-9 postnatal days. An initial delay in axonal loss at P7 (an A_1R- and $A_{2A}R$-mediated effect which can be reinforced by exogenously added ADO) is followed by an A_1R- and $A_{2A}R$-mediated tonic acceleration at P9.

To sum up, the two receptor sets (TrkB and AR) initially delay (P7) axonal loss but promote axonal disconnection at the beginning of the second postnatal week (P9) as mAChRs do. Figure 10 shows a graphic representation of these actions.

However, the experimental groups also differ with respect to the duration of receptor perturbation (two, four, and ten injections at P7, P9, and P15, respectively). Some effects may therefore be due to different durations of receptor inhibition. For instance, compensatory mechanisms may have more or less time to counteract receptor blockade. Thus, it is interesting to evaluate synapse development at different time points after inhibiting receptors for the same time. For the action of some blockers (MET, DPCPX and TrkB-Fc), we probe synapse development at P9 after inhibiting receptors for only two days (from P7-P9). The results show that the three blockers studied significantly reduce the percentatge of the monoinnervated junctions (with respect to the untreated control at P9) independently of their application during four or only two days before the observation at P9. This data reinforce the idea that the receptors role, in normal conditions without the inhibitors, can be to accelerate axonal loss. Interestingly, there are some differences between the blockers. The M_2 blocker MET produces the same effect after four or two days (25.6 ± 1.04 % and 27.2 ± 1.1 % monoinnervated synapses respectively, Fisher's test, $p > 0{,}05$). However, the A_1 blocker DPCPX and the TrkB pathway blocker TrkB-Fc, significantly reduce the monoinnervated synapses even more after two days than after four days of application over the LAL muscle surface (DPCPX: two days 33.7 ± 1.16 %, four days 39.8 ± 1.09 %, $p < 0{,}05$; TrkB-Fc: two days 30.9 ± 1.12 %, four days 42.75 ± 1.07 %, Fisher's test, $p < 0{,}05$). These data reinforce our interpretation that the M_2 receptors start to accelerate axonal elimination around P7 whereas A_1 and TrkB are involved in the initial delay (P5-P7) of axonal loss before shifting to promote axonal disconnection at the beginning of the second postnatal week (P9). The absence of the A_1 and TrkB inhibition between P5-P7 results in a strong effect of the inhibitors when applied between P7-P9.

To assess the overall capacity of the considered signaling on axonal elimination, we investigate the overall effect of prolonged global receptor perturbation on axon number at P15. We studied axon number after more prolonged general block of mAChRs (AT, see above Fig. 3c), ARs (8SPT) and TrkBRs (TrkB-Fc) and found that in all cases, in spite of the continued presence of the inhibitors, monoinnervation is achieved in about 90 % of NMJ at P15 (Fig. 4c; ($p > 0{,}05$, Fisher's test; TrkB-Fc: $n = 825$ NMJs, $N = 3$ mice; 8SPT: $n = 720$ NMJs, $N = 3$ mice)). We conclude that the modulation of axonal competition and the final process of axonal disconnection and loss seems differentially regulated.

Finally, we made some preliminary experiments to show a real cooperation between the receptors. For this purpose we selected receptors which perturbation produces a strong effect on axonal loss at P9 and applied simultaneously their inhibitors in a LAL muscle in four animals. We associated one AR blocker (DPCPX or SCH-58261) with the M_1 blocker PIR. We found that both DPCPX and SCH-58261 add their individual delaying effect on axonal loss to the delaying effect of PIR resulting in 58 % and 36 % respectively less monoinnervated junctions that with PIR only (monoinnervated NMJ after PIR, 39 ± 1.1 %; after PIR + DPCPX, 16.4 ± 1.08 %;

after PIR + SCH-58261, 24.8 ± 0.8 %. The two inhibitor associations differ significatively from the PIR only effect, $p < 0{,}005$).

Postsynaptic receptors cluster during postnatal maturation

mAChR influence on the postsynaptic maturation

We analysed the morphological maturation of the postsynaptic apparatus in the same experimental conditions as those in the previous study on axon loss. The axonal elimination process is accompanied by changes in the morphology of the nicotinic ACh receptor (nAChR) clusters in the postsynaptic component. On the basis of criteria from previous studies on developing mammalian NMJs [23, 26–30], the following maturation stages (MS1–MS4) were defined (Fig. 5a). As normal maturation takes place, changes in the nAChR distribution transform the uniform nAChR oval plaque with an indistinct boundary seen at birth (MS1) into an elongated plaque with a few hints of heterogeneities in receptor density (MS2). This then changes into clusters with small areas of low nAChR density appearing as holes (MS3) that are not innervated. This morphology leads to an increasingly structured pattern of fluorescently labelled independent primary gutters (MS4) below the nerve terminals. Figure 5b shows the percentages of the MS1-MS4 nAChR clusters plotted at days P5-P15.

Stimulation and inhibition of the mAChRs

Figure 6 shows the percentage of MS1-MS4 clusters in the NMJ of the untreated YFP control mice (PBS) and after 2 (P7, Fig. 6a), 4 (P9, Fig. 6b) and 10 (P15, Fig. 6c) applications of the muscarinic substances considered. Figure 7a, b and c also show the percentage of MS3 clusters (postsynaptic clusters in advanced morphological maturation) with one, two or three (or more) axons for each day. This percentage can be taken as an indication of the correspondence between pre- and postsynaptic maturation. After the mAChR antagonists AT, PIR and MET (MT3 does not unambiguously modify the postsynaptic clusters) had been applied for two days, at P7 we found changes in the morphological maturation of the postsynaptic apparatus. Generally there was a high percentage of differentiated MS3 clusters ($p < 0{,}005$, Fisher's test) and fewer MS1 and MS2 ($p < 0{,}005$) (Fig. 6a). Interestingly, many of these MS3 clusters are still innervated by 2–3 axons (Fig. 7a), which indicates some imbalance in the appropiate pre- and postsynaptic correspondence. The fact that postsynaptic maturation accelerates after muscarinic inhibition supports the notion

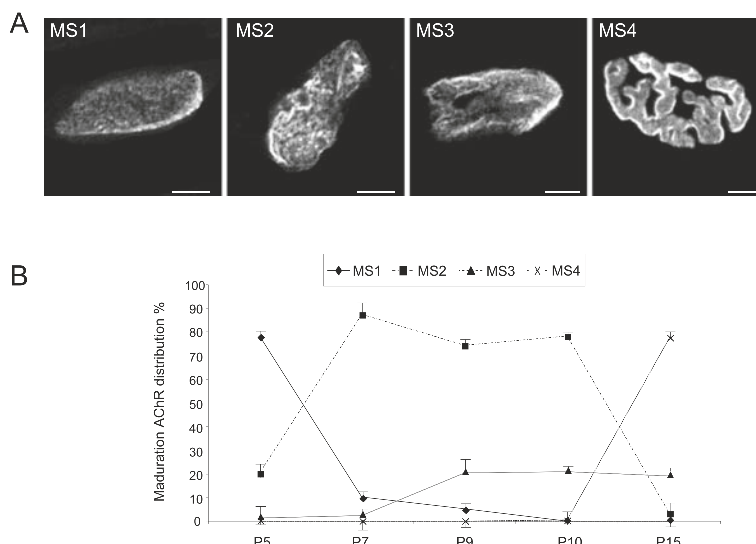

Fig. 5 Postnatal morphological maturation of the postsynaptic apparatus. The axonal elimination process is accompanied by changes in the morphology of the nAChR clusters in the postsynaptic membrane. **a**, the following maturation stages (MS1–MS4) were defined. MS1: Uniform nAChR oval plaque with an indistinct boundary seen in the majority of NMJs at birth. A uniformly distributed porosity can be observed within this plaque. MS2: nAChR elongated oval plaque with a few hints of inhomogeneities in receptor density. The nAChRs are denser on a few narrow ridges that occur within the plaque. MS3: An oval nAChR plaque with one or more fluorescence-free "holes." These holes are not innervated. MS4: The oval nAChR areas have been transformed into a more mature branched pattern with a moderately convoluted external border and high and low receptor density areas. The edge of the holes usually has a high density of receptors. Scale bar: 10 μm. **b**, shows the percentages of the MS1-MS4 nAChR clusters plotted in the interval P5-P15 days

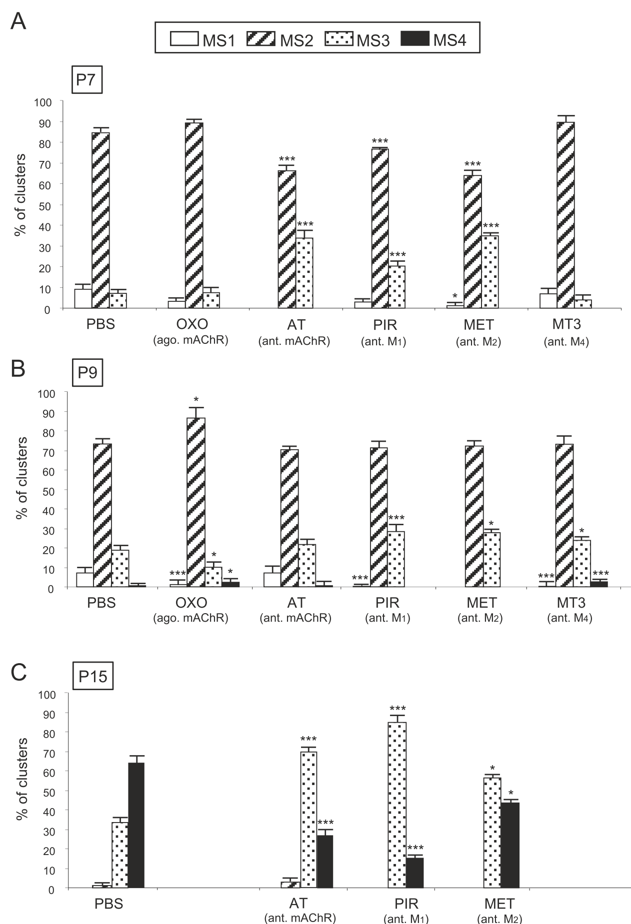

Fig. 6 Maturation of postsynaptic nAChR clusters after stimulation and inhibition of mAChRs. Percentage of MS1-MS4 clusters in the NMJ of untreated YFP control mice (exposed to PBS), and after 2 (P7, in **a**), 4 (P9, in **b**) and in some cases 10 (P15 in **c**) applications of the muscarinic substances considered: OXO, AT, PIR, MET and MT3. Fisher's test * $p < 0,05$, ** $p < 0,01$, *** $p < 0,005$

that in normal conditions (without inhibition) the M_1 and M_2 subtypes have a tonic role and delay maturation. Because OXO does not have a definite significant effect ($p > 0,05$) (Fig. 6a), the tonic muscarinic effect may operate at close to its maximum rate.

At P9, the selective muscarinic drugs PIR, MET and MT3 accelerated cluster maturation, and produced fewer MS1 and more MS3 clusters (Fisher's test; for MS3: PIR ($p < 0,005$); MET i MT3 ($p < 0,05$); even MS4 for MT3; see Fig. 6b), many of which (for AT, PIR and MET experiments) were innervated by 2–3 axons as they were at P7 (Fig. 7b). This also indicates that at P9 the M_1, M_2 and M_4 subtypes are involved in delaying the normal maturation process in normal conditions. However, AT does not change the normal percentage of the cluster subtypes (though the MS3 subtype is also innervated by 2–3 axons, $p > 0,05$) and OXO moderately accelerates maturation (by reducing MS1 ($p < 0,005$) and increasing the MS2 subtype, $p < 0,01$). Thus, the use of the subtype-unselective drugs AT and OXO reveal the complex involvement of the mAChRs in the morphological maturation process of the postsynaptic receptor clusters. The coincident contribution of other signalling will be considered below.

With the unselective mAChR antagonist AT and the selective M_1 and M_2 muscarinic inhibitors, and specially with PIR, we observed at P15 that postsynaptic maturation seems to be slower and partially retained at the MS3 subtype (Fig. 6c) though most MS3 are

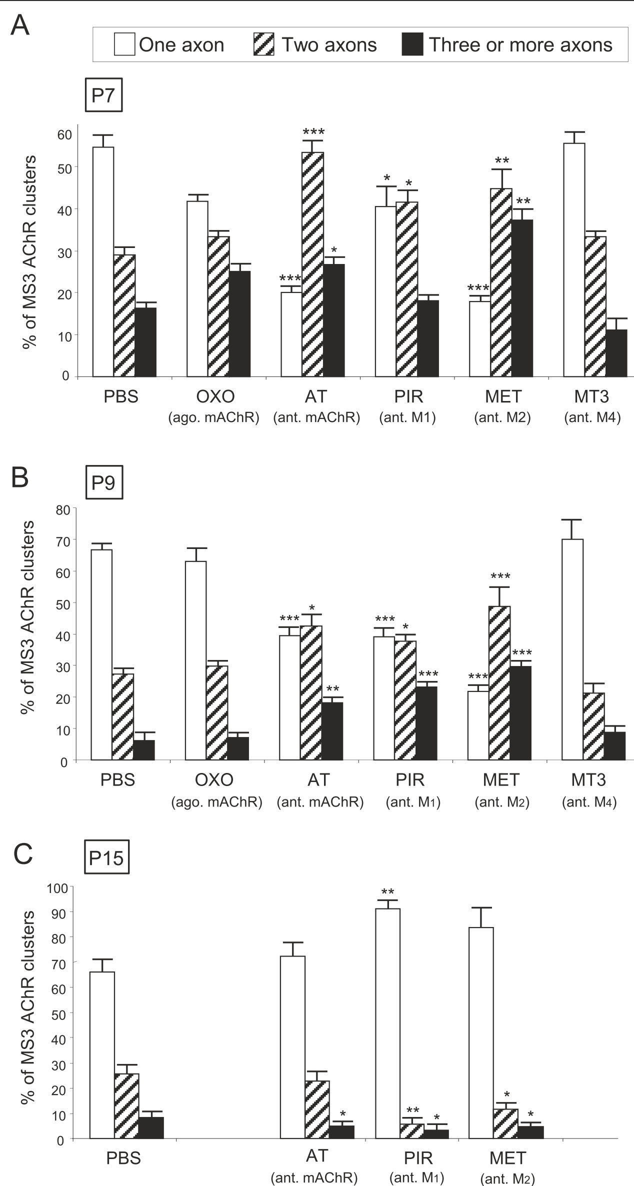

Fig. 7 Pre- and postsynaptic maturation in the MS3 clusters after stimulation and inhibition of the mAChRs. For each day considered (P7 in **a**, P9 in **b** and P15 in **c**) the figure shows the percentage of MS3 clusters (the oval nAChR plaques with fluorescence-free holes that mature at a faster rate) with one, two and three or more axons as an indication of the appropiate correspondence of the pre- and postsynaptic maturation. Fisher's test * $p < 0{,}05$, ** $p < 0{,}01$, *** $p < 0{,}005$

already monoinnervated in the presence of AT, PIR and MET (Fig. 7c).

Thus, as far as postsynaptic clusters are concerned, in normal conditions mAChRs tend to produce some delay in maturation throughout the P5-P9 period and this effect is extended at P15 when axonal elimination is almost complete whether muscarinic modulators are used or not.

Other signalling mechanisms involved in postsynaptic maturation

Figure 8a shows that after two days of using TrkB-Fc to sequester endogenous BDNF/NT-4, nAChR maturation is delayed at P7 because of the persistence of many MS1 clusters ($p < 0,005$, Fisher's test). This indicates that the normal stimulation of the TrkB pathway promotes postsynaptic maturation at around P7. This tendency is reversed at P9 after four days of exposure to TrkB-Fc because of the clear increase in the MS3 subtype with respect to the untreated control ($p < 0,05$, Fig. 8b). In addition, many of these MS3 clusters are polyinnervated (with three or more axons, $p < 0,005$, Fig. 9b). Ten applications (one application every day after P5) of

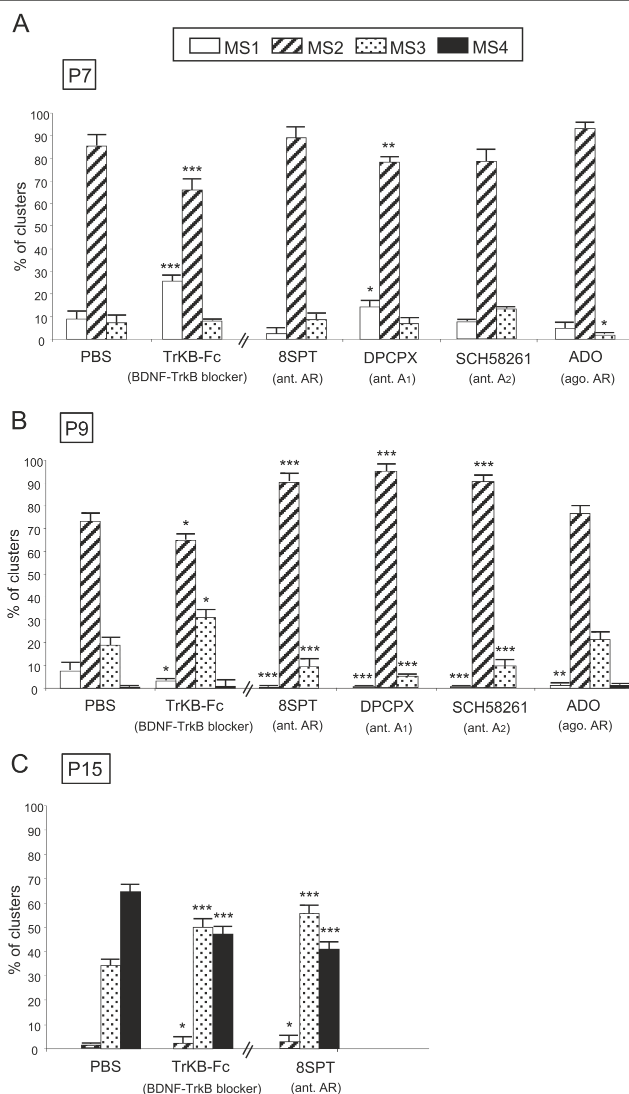

Fig. 8 Involvement of the AR and TrkB receptors in the morphological maturation of the postsynaptic apparatus. The figure shows the percentage of the MS1-MS4 clusters in the NMJ of the untreated YFP control mice (exposed to PBS), and after 2 (P7 in **a**), 4 (P9, in **b**) and in some cases 10 (P15, in **c**) applications (one application every day after P5) of the TrkB blocking chimera TrkB-Fc, the AR pan-inhibitor 8SPT and the AR agonist ADO. We also studied axonal elimination after selectively blocking A_1R with DPCPX and inhibiting $A_{2A}R$ with SCH-58261. Fisher's test: * $p < 0,05$, ** $p < 0,01$, *** $p < 0,005$

TrkB-Fc reveal some delay of the postsynaptic maturation at P15 (increased MS3 and less MS4 clusters, Fig. 8c). Thus, the TrkB pathway seems to have a complex effect on postsynaptic maturation (accelerated at P7, delayed at P9 and accelerated once again thereafter).

With regard to the AR pathway, at P7 we found that the unselective antagonist 8SPT had no effect on the maturation of postsynaptic clusters ($p > 0{,}05$, Fisher's test Fig. 8a) although when 8SPT was applied in the period P5-P8 (observation at P9, Fig. 8b) MS2 clusters increased and MS1 and MS3 clusters decreased ($p < 0{,}005$), which indicates

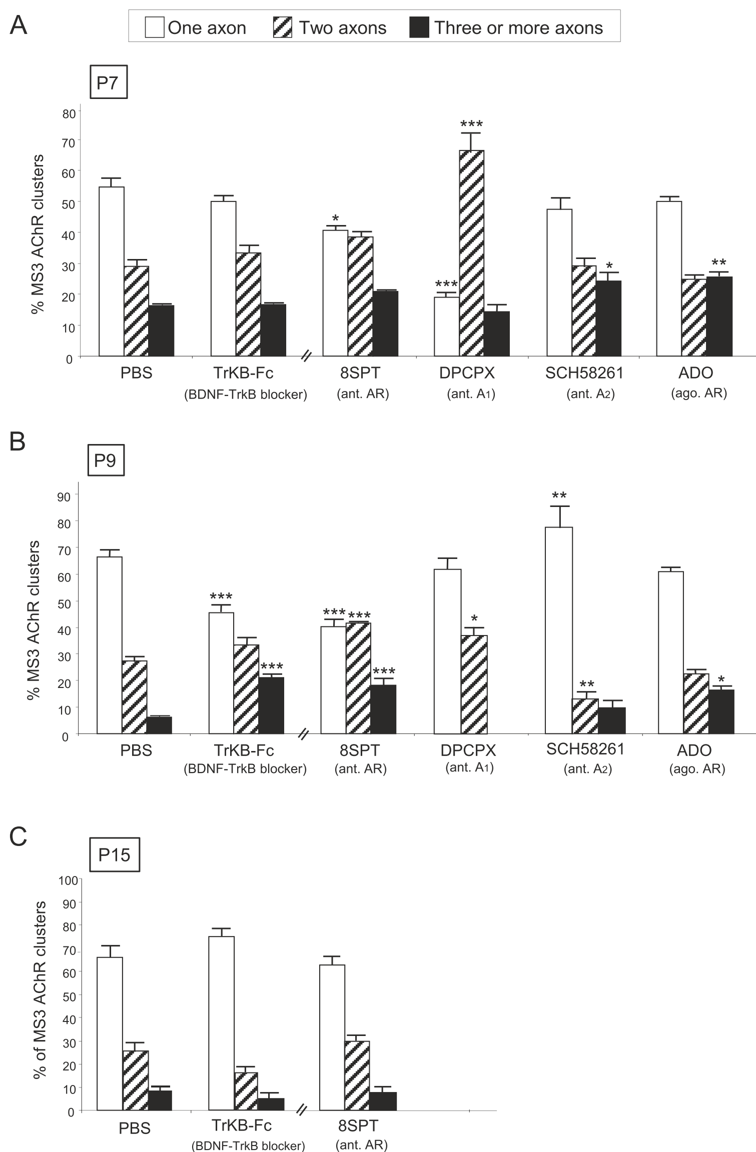

Fig. 9 Pre- and postsynaptic maturation in the MS3 clusters. AR and TrkB pathways modification. For each day considered (P7 in **a**, P9 in **b** and P15 in **c**), the figure shows the percentage of MS3 clusters with one, two and three or more axons as an indication of the appropiate correspondence of the pre- and postsynaptic maturation. Fisher's test: * $p < 0{,}05$, ** $p < 0{,}01$, *** $p < 0{,}005$

some delay in the transition from MS2 to MS3. Also, many of the few MS3 clusters remain polyinnervated with two or three axons ($p < 0{,}005$, Fig. 9b). Interestingly, we observed that after daily applications of 8SPT between P5 and P15 the postsynaptic maturation seems to be partially retained at the MS3 subtype (Fig. 8c). Thus, AR in normal conditions without inhibition can accelerate maturation somewhat during the P7-P9 period. Interestingly, exposure of the LAL muscle to the agonist ADO does not unambiguously change the normal distribution of the clusters at P7 (although it decreases MS3 slightly and a number of these clusters are innervated by three or more axons [$p < 0{,}01$, Figs. 8a and 9a]) and P9 (although there is a slight decrease in MS1, $p < 0{,}01$). This indicates that the tonic effect of the AR manifested by using 8SPT can not be clearly changed with exogenously added agonist. Which AR subtypes are involved in the tonic effect of endogenous ADO? We analysed the maturation of nAChR clusters after selective block of A_1R with DPCPX or $A_{2A}R$ block with SCH-58261. Our data indicate that blocking A_1R at P7 and both A_1R and $A_{2A}R$ at P9 delays the maturation of normal clusters meaning that both receptor subtypes can accelerate postsynaptic maturation in normal conditions.

The diagram in Fig. 10 is a graphic representation of the influence of the mAChRs, and the AR and TrkB receptors on postnatal axonal elimination and postsynaptic maturation.

Discussion

The main observation of the present study is that the coordinated action of the mAChRs (M_1, M_2 and M_4), AR (A_1R and $A_{2A}R$) and TrkB signalling modulates the conditions of axonal competition and promotes (around P7-P9) the disconnection of supernumerary nerve endings.

Presynaptic mAChRs M_1-, M_2- and M_4-subtypes modulate axonal loss

Although there is not agreement about whether all mAChR subtypes are present in the NMJ [25, 31], some of these receptors play a role in ACh release both during development [18, 21, 32] and in the adult [32, 33]. In P6-P7 mice, we observed that M_1 and M_4 subtypes are involved in a mechanism that delays axonal elimination. However, the action of all muscarinic receptors as a whole indicates that the muscarinic mechanism increases the rate of axonal loss in dual junctions and, therefore, the final transition to the monoinnervation. It seems that NMJs with different maturation levels have different sensitivities to muscarinic regulation. The relative levels of these receptors or differences in turnover rate may contribute to the different effects observed. Using Western blotting we observed that in the adult, M_1, M_2, M_3 and M_4 receptors are more abundant than in the newborn [25]. In fact, changes in the expression of muscarinic receptors during development have been described in embryonic chick heart and retina [34], in carotid body, petrosal and superior cervical ganglion of the cat [35] and in rat brain [36]. In addition there are differences in the internalization and turnover of the mAChR family members [37, 38] and endocytosis may favour the coupling of the receptors to different signal transduction cascades [39].

However, the M_1-M_2 subtype pair (in substitution of the M_1-M_4 pair) cooperates to favour the full sequence of synapse elimination at P9 (the three-to-one axon transition). The delayed two-to-one transition induced by AT at P7 (which reveals accelerated axonal elimination in normal conditions without the inhibitor) may be interpreted as an early manifestation of the consistent mAChR-modulated axonal loss that is fully manifested at P9. The muscarinic mechanism appears to operate at close to maximum capacity and, therefore, may not be able to increase its efficacy beyond P7 with agonists like OXO. Interestingly, in spite of the continued presence of the M_1 and M_2 inhibitors, the elimination process comes to its normal conclusion at the end of the second postnatal week (P15). This suggests that other signaling mechanisms help to resolve the correct synaptic connectivity. Alternatively, M_1 and M_2 signaling may be not required at

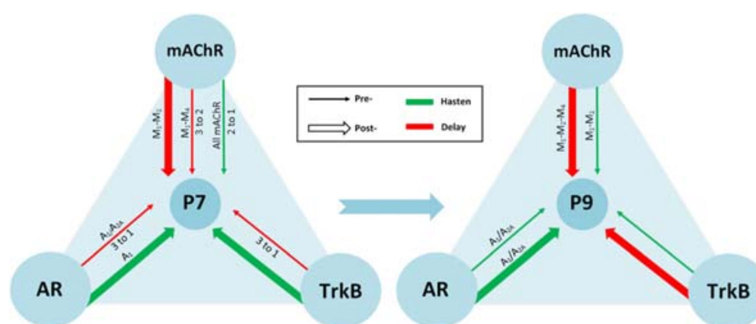

Fig. 10 Influence of the mAChR, AR and TrkB receptors on postnatal axonal elimination and synaptic maturation

all for the final step of axonal elimination because the receptor inhibitors produce only transient perturbations in elimination but axon los is completed normally around P15. Our interpretation is that all considered receptors (see later) intervene in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the lossers but, thereafter, the time and conditions of the final elimination would occur with some autonomy.

In summary, the results show that a tonic muscarinic mechanism initially delays axonal elimination (a selective M_1-M_4 effect). However, the overall mAChR effect may accelerate the last phase of axonal disconnection, the two-to-one transition. Thereafter the muscarinic effect at around P9 clearly promotes elimination of all supernumerary nerve terminals (an M_1-M_2 effect).

Which mAChR subtypes couple to regulate ACh release? In the mature NMJ, M_1 and M_2 mAChRs modulate evoked transmitter release by positive and negative feedbacks, respectively [13, 40]. M_2 is more prevalent [20, 31]. During synaptogenesis, [13, 14, 41], in the monoinnervated junctions and the strong terminal in dually innervated junctions both M_1 and M_2 are coupled to potentiate ACh release. However, in the weakest nerve terminal in dual junctions only M_2 potentiates release whereas M_1 and M_4 couple to inhibit ACh secretion. A mAChR-PKC-VDCC cascade is involved in controlling ACh release in the weak ending. Blocking PKC, VDCCs (P/Q-, N- or L-type or Ca^{2+} influx) or mAChRs (M_1- and/or M_4-subtypes) can lead to similar percentage increases in the size of the synaptic potentials evoked by weak axons [15–17].

How are related the release capacity of the strong and weak endings and the loss of axons described here? At P7, the release capacity of the weakest endings was increased by the inhibitors PIR and MT3, whereas ACh release from the strong ending was reduced or unaffected [16]. Thus, the difference in ACh release between the strong and weak nerve endings is reduced, and this fact may change the competitive conditions of the nerve terminals. We do not know exactly how is the ACh release capacity of the weak and strong endings in the LAL muscle at P9. However, between P7 and P9, the percentage of polyinnervated junctions changes only by about 10%. The configuration of mAChR in the monoinnervated synapses is not mature until P15 [13], which suggests that the competitive interactions between axons peak at around P9 and their release capacity is probably not very different from what it is at P7. If this is so, the reduction of the competitive advantage and disadvantage linked to ACh release of the strong and weak endings produced by PIR and the reduccion of the strength of the different axons produced by MET (MT3 does not play at P9) may considerably delay axonal loss. We found here that this is the case.

Contribution of AR and TrkB receptor pathways

Several data suggest the involvement of other receptors. The mAChR agents alter the time course of the synapse elimination but not its final chronology. Experimental manipulations of the PKC/PKA pathways can also change the time course but not the final conclusion of synapse elimination [23, 32, 42, 43]. This indicates that different receptors with their intracellular mechanisms can be used in the process of synapse elimination.

AR are present in the motor terminals of the newborn and adult NMJs [44, 45]. These receptors can collaborate with mAChR to reduce depression during repetitive activity [44, 46, 47]. During development, low extracellular concentrations of ADO may activate both A_1R and $A_{2A}R$ and have a facilitatory action on ACh release [48]. Our results show that mAChR and AR delay axonal loss at P7 (although mAChR accelerate the last phase of axonal disconnection) but accelerate it at P9. The results showing an additive effect between M_1 and A_1 or A_{2A} are an indication of the cooperation between at least these receptors.

The BDNF-TrkB pathway also plays a biphasic role. Judging from the effect of the TrkB-IgG chimera, BDNF initially delays elimination and subsequently accelerates it. Neurotrophins and their receptors in muscle and nerve are expressed in both development and adulthood [49–54]. Low doses of BDNF rapidly induce a TrkB-dependent potentiation at developing NMJs in culture. In developing muscles, BDNF increases ACh release in both the weak and strong endings at P6-P7 [55]. In addition, exogenous BDNF increases the percentage of functional polyinnervated junctions [17]. Interestingly, exogenous BDNF infusion delayed synapse elimination in the mouse LAL muscle [56] The delaying effect of the TrkB pathway on axonal elimination at P7 described here may be releated with the BDNF potentiation of the weakest endings about to be eliminated. However, blocking the TrkB receptor or neutralizing endogenous BDNF with the TrkB-IgG chimera at P7 does not affect the quantal content of the weak endings but increases release in the strong ending, which suggests that endogenous BDNF, in this developmental period, may surprisingly reduce release in the strongest ending [55]. The delaying effect of the TrkB pathway on axonal elimination at P7 may be related to the BDNF-mediated lesser release and presumed lesser competitive force of the strong axon. The TrkB pathway accelerate elimination at P9. The progressive maturation of the NMJ at P9 may change the operation conditions of the BDNF-TrkB pathway in the strongest endings resulting in more efficient competition and axonal elimination [55, 57].

Relation between mAChR, AR and TrkB pathways

The mAChR, AR and TrkB pathways share a link mediated by the set phospholipase C (PLC)-phosphatidylinositol 4,5-bisphosphate (PIP2)-diacylglycerol (DAG)-protein kinase C

(PKC), which modulates P/Q-type VDCC [40, 58]. Interestingly, PKC couples to potentiate ACh release in the adult monoinnervated NMJ, in the strong ending in developing dual junctions and in the solitary ending of the recently monoinnervated junctions at the end of developmental maturation. However, reduces release in the weakest axons in dual junctions and, therefore, PKC may be determinant in the regulation of axonal loss [18].

Involvement of the mAChR, AR and TrkB in the maturation of nAChR clusters

mAChR [25], AR [44] and TrkB receptors [55] are present in the postsynaptic site of NMJs and are involved in organizing them [50, 59–61]. The changes we observed may be caused by the pharmacological tools directly acting on these receptors, as a side-effect of a primary effect on the axonal elimination rate or a combination of the two mechanisms. The first change in synapse elimination may be a reduction in the quantal efficacy because of a local decrease in nAChR density [62]. This postsynaptic change may begin before the overlying axon withdraws [63]. However, polyneuronal innervation decreases considerably at a time when relatively few postsynaptic nAChR are lost [10, 23]. We found here that several situations of increased axon loss or retention do not coincide with the maturation of the nAChR clústers, which suggests independent regulation. Interestingly, prolonged M_1 and M_2 inhibition results in a defect in postsynaptic maturation at P15. Especially, M_1 perturbation had a strong effect. This finding suggests a requirement for M_1 and M_2 signaling in postsynaptic maturation and occurs when axon loss has been completed. In addition, AR block with 8SPT and TrkB pathway block with the TrkB-Fc chimera, similarly delay postsynaptic maturation at P15 (in all cases less MS4 mature nAChR clusters) indicating also the need of these signalling pathways in postsynaptic maturation. Selective nAChR-phosphorylation by PKC and PKA is one of the causes of nAChR dispersion and stability, respectively [64–66]. An activity-dependent coordinated mAChR-AR-TrkB effect on these postsynaptic kinases could be a key mechanism in NMJ maturation.

Conclusion

Synaptic contacts are lost throughout the nervous system during both histogenesis and ageing and experience-dependent neuronal plasticity requires maintenance of newly formed synapses, while others are eliminated. We investigate the involvement of muscarinic, purinergic and neurotrophin receptor signaling in developmental synapse elimination. The three receptor sets intervene in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the lossers but, thereafter, the final elimination would occur with some autonomy and independently of postsynaptic maturation.

Methods

Animals

Transgenic B6.Cg-Tg (Thy1-YFP)16 Jrs/J mice were used (The Jackson Laboratory). Transgenic mice express spectral variants of GFP (yellow-YFP) at high levels in motor and sensory neurons, as well as in subsets of central neurons. Axons are brightly fluorescent all the way to the terminals. No expression is detectable in nonneural cells. All experiments were conducted on Thy1-YFP-expressing mice. In some cases, we check our results with C57BL/6J mice (The Jackson Laboratory).

Experiments were performed on the *Levator auris longus* (LAL). Neonatal pups of either sex (4–30 days) were obtained and the date of birth was designated postnatal day 0 (P0). We minimized the variability in our measurements by carefully monitoring the timing of conception. Also, the weights of the individuals were within 5 % of the mean for a given day after conception. The mice were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All experiments on animals have been reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 0233).

Injection procedure

The newborn mice were anesthetized with 2 % tribromoethanol (0.15 ml/10 g body weight, i.p.). Under aseptic conditions, various solutions (antagonists and agonists of the considered receptors) were administered in 50 μl of sterile physiological saline or dimethyl sulfoxide (DMSO) by subcutaneous injection over the LAL external surface as described elsewhere [22]. The animals received 2, 4 or 10 injections from postnatal day 5, and the LAL muscles were studied on days 7, 9 and 15. The solutions were administered at a concentration in accordance with the previously reported biological action of the substance [14, 55, 67].

Tissue preparation and histochemistry

Neonatal pups were given a lethal dose of 2 % tribromoethanol. Their heads were removed and fixed in 4 % paraformaldehyde for 1.5 h. After washing in phosphate-buffered saline (PBS), LAL muscles were removed and post-fixed for 45 minutes. After washing in PBS, Thy1-YFP LAL muscles were incubated in PBS containing a 1/800 dilution of 1μg/ml tetramethylrhodamine conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) for 1h at room temperature.

Double immunofluorescence and confocal analysis were performed on the C57BL/6J LAL muscle. Whole mounts of LAL were processed to detect the axons with

an antibody against 200-kD neurofilamentprotein and postsynaptic nicotinic acetylcholine receptors (nAChRs) with TRITC-α- BTX (Molecular Probes, Eugene, OR). Muscles were incubated overnight only with the rabbit antibody against 200-kD neurofilament (1:1,000; Sigma) in 1 % bovine serum albumin (BSA). The appropriate secondary antibody (conjugated with Alexa-fluor 488) donkey anti-rabbit (Molecular Probes) was added and incubated for 4 h. The antibody specificity was tested by incubation in the absence of primary antibody. At least three muscles were used as negative controls (not shown). Whole muscles were mounted in Mowiol with p-phenylenediamide (Sigma).

Morphological analysis and Confocal microscopy

NMJs were analyzed using an inverted Nikon TE-2000 fluorescent microscope (Nikon, Tokyo, Japan) connected to a personal computer running image analysis software (ACT-1, Nikon). The number of axons per endplate was counted. Because of the difficulty in determining the exact number of axonal inputs for each nAChR cluster when more than two axons converged at the same synaptic site, we classified the NMJs into three groups only: junctions that were monoinnervated, doubly innervated, or innervated by three or more terminal axons. These data enabled us to calculate the "average number of axonal inputs" and the "percentage of polyneuronal innervation" for all fibers receiving two or more axons.

In PBS, DMSO control experiments or untreated animals, we determined the number of axons per endplate between days 5 to 30 and the postsynaptic nAChR cluster morphology on days 5, 7, 9, 10 and 15. Animals were injected with PBS or DMSO over the LAL muscle. The injections were performed from day 5 and the animals sacrificed on days 7, 9, and 15. No differences were found between the muscles injected or not with PBS, either in the nAChR cluster morphology or the number of axons per endplate, thus indicating that the injection procedure did not in itself induce changes in the overall morphology of the motor endplate and nerve terminals. The final concentration of DMSO in control and drug-treated preparations was 0.1% (v/v). In control experiments, this concentration of DMSO did not affect any of the parameters studied (data not shown).

To determine the effect of different treatments on the maturity of nAChR clusters at the NMJ during the period in which polyneuronal innervation is being eliminated, the maturation of the clusters was divided into four morphological stages (MS1–MS4) on the basis of criteria from previous studies of developing mammalian NMJs [23, 26, 27, 29] (Fig. 5). MS1: Uniform nAChR oval plaque with an indistinct boundary seen in the majority of NMJs at birth. A uniformly distributed porosity can be observed within this plaque. MS2: nAChR elongated oval plaque with a few hints of inhomogeneities in receptor density. The nAChRs are denser on a few narrow ridges within the plaque. MS3: An oval nAChR plaque with one or more fluorescence-free "holes." These holes are not innervated. MS4: The oval nAChR areas have been transformed into a more mature branched pattern with a moderately convoluted external border and high and low receptor density areas. The edge of the holes usually has a high density of receptors. High-resolution confocal images were obtained with a 63x oil objective (1.4 numerical aperture) on a Nikon TE-2000 confocal microscope. Z stacks were obtained at 0.5-μm step size for depths of 20–40 μm, and additional optical sections above and below each junction were collected to ensure that the entire synapse was included.

Statistical analysis

All NMJs visible in their entirety were scored, with a minimum of 100 per muscle. At least six muscles were studied for each age and condition examined. Fisher's test was applied to compare percentages. The criterion for statistical significance was $p < 0.05$. The categories were scored and the counting was performed by a person with no knowledge of the age or treatment of the animals. The data are presented as mean ± SD.

Drugs

Purinergic agents

Non-selective AR agonists and antagonists The stock solution of adenosine 5′-triphosphate disodium salt hydrate (ADO; A9251, Sigma-Aldrich, St. Louis, MO) was made up as a 100 mM solution in deionized water. The stock solution of 8-(p-sulfophenyl)theophylline (8-SPT; A013, Sigma-Aldrich, St. Louis, MO) was made up as a 100 μM solution also in deionized water. The working solutions were adenosine (25μM) and 8-SPT (100 μM).

Selective A_1 R and A_{2A} R antagonists The stock solutions were 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; C101, Sigma-Aldrich) 50 mM, and 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e] [1, 2, 4] triazolo[1,5-c]pyrimidin-5-amine (SCH-58261; 2270, Tocris, Bristol, UK) 100 mM, both dissolved in DMSO. Working solutions were DPCPX (100 nM) and SCH-58261 (50 nM).

Muscarinic agents

Non-selective mAChR agonists and antagonists The stock solutions were oxotremorine M (OXO; O100, Sigma - Aldrich, St. Louis, MO) 50 mM; atropine (AT; A0132, Sigma - Aldrich, St. Louis, MO) 200 μM both dissolved in deionized water. Working solutions were oxotremorine M (1μM) and atropine (2μM).

Selective M_1, M_2 and M_4 mAChR antagonists The stock solutions were pirenzepine dihydrochloride (PIR; 1071, Tocris Bioscience) 10 mM; methoctramine (MET; M105, Sigma – Aldrich, St. Louis, MO) 1 mM; muscarinic toxin 3 (MT-3; M-140, Alomone Labs) 50 μM. The working solutions used were pirenzepine (10 μM), methoctramine (1μM), and muscarinic toxin 3 (100 nM).

TrkB receptor-related agent

The following stock solutions was used: recombinant human trkB/Fc Chimera (trkB-Fc; 688-TK;R&D Systems), 100μg/ml. Working solution was trkB-Fc 5μg/ml.

Abbreviations

A_1R, A_1-type receptors; $A_{2A}R$, A_{2A}-type receptors; ACh, acetylcholine; ADO, adenosine; AR, adenosine receptors; BDNF, Brain-derived neurotrophic factor; BSA, bovine serum albumin; DMSO, Dimethyl sulfoxide; LAL, Levator auris longus muscle; M_1, M_1-type muscarinic acetylcholine receptor; M_2, M_2-type muscarinic acetylcholine receptor ; M_4, M_4-type muscarinic acetylcholine receptor; mAChR, muscarinic acetylcholine receptor; MS, maduration stage; nAChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; NT-4, neurotrophin-4 PBS, phosphate-buffered saline; NTR, neurotrophin receptors; PKA, protein kinase A; PKC, protein kinase C; SD, Sprague-Dawley; TrkB, tropomyosin-related kinase B receptor; VDCC, voltage-dependent calcium channels

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Availability of data and material

We think that our data are not appropriate for the available repository database in neuroscience.

Authors' contributions

L.N: data collection, quantitative analysis; literature search, data interpretation,; E.H, AS and M.T: data collection, quantitative analysis; M.S: technical suport; N.G: statistics; J.T., N.G and M.A.L,: conception and design, literature search, data interpretation, manuscript preparation. J.T., N.G and M.A.L. contributed equally to this work. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing insterests.

Ethics approval and consent to participate

The mice were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All experiments on animals have been reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 0233).

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References

1. Jansen JK, Fladby T. The perinatal reorganization of the innervation of skeletal muscle in mammals. Progress in Neurobiology. 1990;34:39–90.
2. Sanes JR, Lichtman JW. Development of the vertebrate neuromuscular junction. Annual Review of Neuroscience. 1999;22:389–442.
3. Purves D, Lichtman JW. Elimination of synapses in the developing nervous system. Science. 1980;210:153–7.
4. Chen C, Regehr WG. Developmental remodeling of the retinogeniculate synapse. Neuron. 2000;28:955–66.
5. Ribchester RR, Barry JA. Spatial versus consumptive competition at polyneuronally innervated neuromuscular junctions. Experimental Physiology. 1994;79:465–94.
6. Liu Y, Fields RD, Festoff BW, Nelson PG. Proteolytic action of thrombin is required for electrical activity-dependent synapse reduction. Proceedings of the National Academy of Sciences of the United States of America. 1994;91:10300–4.
7. Nguyen QT, Lichtman JW. Mechanism of synapse disassembly at the developing neuromuscular junction. Current Opinion in Neurobiology. 1996;6:104–12.
8. Chang Q, Balice-Gordon RJ. Nip and tuck at the neuromuscular junction: a role for proteases in developmental synapse elimination. BioEssays. 1997;19:271–5.
9. Herrera AA, Zeng Y. Activity-dependent switch from synapse formation to synapse elimination during development of neuromuscular junctions. Journal of Neurocytology. 2003;32:817–33.
10. Nelson PG, Lanuza MA, Jia M, Li M-X, Tomas J. Phosphorylation reactions in activity-dependent synapse modification at the neuromuscular junction during development. Journal of Neurocytology. 2003;32:803–16.
11. Wyatt RM, Balice-Gordon RJ. Activity-dependent elimination of neuromuscular synapses. Journal of Neurocytology. 2003;32:777–94.
12. Buffelli M, Busetto G, Bidoia C, Favero M, Cangiano A. Activity-dependent synaptic competition at mammalian neuromuscular junctions. News in Physiological Sciences. 2004;19:85–91.
13. Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J. Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. The European Journal of Neuroscience. 2003;17:119–27.
14. Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J. Muscarinic autoreceptors related with calcium channels in the strong and weak inputs at polyinnervated developing rat neuromuscular junctions. Neuroscience. 2004;123:61–73.
15. Santafé MM, Lanuza MA, Garcia N, Tomàs M, Tomàs J. Coupling of presynaptic muscarinic autoreceptors to serine kinases in low and high release conditions on the rat motor nerve terminal. Neuroscience. 2007;148:432–40.
16. Santafe MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Tomàs J. Presynaptic muscarinic receptors, calcium channels, and protein kinase C modulate the functional disconnection of weak inputs at polyinnervated neonatal neuromuscular synapses. Journal of Neuroscience Research. 2009;87:1195–206.
17. Tomàs J, Santafé MM, Lanuza MA, García N, Besalduch N, Tomàs M. Silent synapses in neuromuscular junction development. Journal of Neuroscience Research. 2011;89:3–12.
18. Santafé MM, Garcia N, Lanuza MA, Tomàs M, Tomàs J. Interaction between protein kinase C and protein kinase A can modulate transmitter release at the rat neuromuscular synapse. Journal of Neuroscience Research. 2009;87:683–90.
19. Keller-Peck CR, Feng G, Sanes JR, Yan Q, Lichtman JW, Snider WD. Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. The Journal of Neuroscience. 2001;21:6136–46.
20. Tomàs J, Santafé MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Obis T, Priego M, Hurtado E. Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. Journal of Neuroscience Research. 2014;92:543–54.
21. Santafé MM, Garcia N, Lanuza MA, Uchitel OD, Tomás J. Calcium channels coupled to neurotransmitter release at dually innervated neuromuscular junctions in the newborn rat. Neuroscience. 2001;102:697–708.
22. Lanuza MA, Garcia N, Santafe M, Nelson PG, Fenoll-Brunet MR, Tomas J. Pertussis toxin-sensitive G-protein and protein kinase C activity are involved in normal synapse elimination in the neonatal rat muscle. Journal of Neuroscience Research. 2001;63:330–40.
23. Lanuza MA, Garcia N, Santafé M, González CM, Alonso I, Nelson PG, Tomàs J. Pre- and postsynaptic maturation of the neuromuscular junction during neonatal synapse elimination depends on protein kinase C. Journal of Neuroscience Research. 2002;67:607–17.
24. Santafé MM, Sabaté MM, Garcia N, Ortiz N, Lanuza MA, Tomàs J. Anti-GM2 gangliosides IgM paraprotein induces neuromuscular block without neuromuscular damage. Journal of Neuroimmunology. 2008;204:20–8.
25. Garcia N, Santafé MM, Salon I, Lanuza MA, Tomàs J. Expression of muscarinic acetylcholine receptors (M1-, M2-, M3- and M4-type) in the

neuromuscular junction of the newborn and adult rat. Histology and Histopathology. 2005;20:733–43.
26. Steinbach JH. Developmental changes in acetylcholine receptor aggregates at rat skeletal neuromuscular junctions. Developmental Biology. 1981;84:267–76.
27. Slater CR. Neural influence on the postnatal changes in acetylcholine receptor distribution at nerve-muscle junctions in the mouse. Developmental Biology. 1982;94:23–30.
28. Slater CR. Postnatal maturation of nerve-muscle junctions in hindlimb muscles of the mouse. Developmental Biology. 1982;94:11–22.
29. Bewick GS, Young C, Slater CR. Spatial relationships of utrophin, dystrophin, beta-dystroglycan and beta-spectrin to acetylcholine receptor clusters during postnatal maturation of the rat neuromuscular junction. Journal of Neurocytology. 1996;25:367–79.
30. Garcia N, Tomàs M, Santafe MM, Lanuza MA, Besalduch N, Tomàs J. Blocking p75 (NTR) receptors alters polyinnervationz of neuromuscular synapses during development. Journal of Neuroscience Research. 2011;89:1331–41.
31. Wright MC, Potluri S, Wang X, Dentcheva E, Gautam D, Tessler A, et al. Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses. The Journal of Neuroscience. 2009;29:14942–55.
32. Santafé MM, Garcia N, Lanuza MA, Tomàs J. Protein kinase C activity affects neurotransmitter release at polyinnervated neuromuscular synapses. Journal of Neuroscience Research. 2007;85:1449–57.
33. Santafé MM, Lanuza MA, Garcia N, Tomàs J. Calcium inflow-dependent protein kinase C activity is involved in the modulation of transmitter release in the neuromuscular junction of the adult rat. Synapse. 2005;57:76–84.
34. McKinnon LA, Nathanson NM. Tissue-specific regulation of muscarinic acetylcholine receptor expression during embryonic development. The Journal of Biological Chemistry. 1995;270:20636–42.
35. Bairam A, Joseph V, Lajeunesse Y, Kinkead R. Developmental pattern of M1 and M2 muscarinic gene expression and receptor levels in cat carotid body, petrosal and superior cervical ganglion. Neuroscience. 2006;139:711–21.
36. Tice MA, Hashemi T, Taylor LA, McQuade RD. Distribution of muscarinic receptor subtypes in rat brain from postnatal to old age. Brain Research Developmental Brain Research. 1996;92:70–6.
37. Reiner C, Nathanson NM. Muscarinic receptor trafficking. Handbook of Experimental Pharmacology. 2012;208:61–78.
38. Ockenga W, Tikkanen R. Revisiting the endocytosis of the M2 muscarinic acetylcholine receptor. Membranes. 2015;5:197–213.
39. Pierce KL, Luttrell LM, Lefkowitz RJ. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene. 2001;20:1532–9.
40. Santafé MM, Lanuza MA, Garcia N, Tomàs J. Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. The European Journal of Neuroscience. 2006;23:2048–56.
41. Santafé MM, Garcia N, Lanuza MA, Uchitel OD, Salon I, Tomàs J. Decreased calcium influx into the neonatal rat motor nerve terminals can recruit additional neuromuscular junctions during the synapse elimination period. Neuroscience. 2002;110:147–54.
42. Jia M, Li M, Dunlap V, Nelson PG. The thrombin receptor mediates functional activity-dependent neuromuscular synapse reduction via protein kinase C activation in vitro. Journal of Neurobiology. 1999;38:369–81.
43. Nelson PG. Activity-dependent synapse modulation and the pathogenesis of Alzheimer disease. Current Alzheimer Research. 2005;2:497–506.
44. Garcia N, Priego M, Obis T, Santafe MM, Tomàs M, Besalduch N, Lanuza MA, Tomàs J. Adenosine A1 and A2A receptor-mediated modulation of acetylcholine release in the mice neuromuscular junction. The European Journal of Neuroscience. 2013;38:2229–41.
45. Garcia N, Priego M, Hurtado E, Obis T, Santafe MM, Tomàs M, Lanuza MA, Tomàs J. Adenosine A2B and A3 receptor location at the mouse neuromuscular junction. Journal of Anatomy. 2014;225:109–17.
46. Correia-de-Sá P, Sebastião AM, Ribeiro JA. Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. British Journal of Pharmacology. 1991;103:1614–20.
47. Santafé MM, Garcia N, Tomàs M, Obis T, Lanuza MA, Besalduch N, Tomàs J. The interaction between tropomyosin-related kinase B receptors and serine kinases modulates acetylcholine release in adult neuromuscular junctions. Neuroscience Letters. 2014;561:171–5.
48. Pousinha PA, Correia AM, Sebastião AM, Ribeiro JA. Predominance of adenosine excitatory over inhibitory effects on transmission at the neuromuscular junction of infant rats. The Journal of Pharmacology and Experimental Therapeutics. 2010;332:153–63.
49. Funakoshi H, Belluardo N, Arenas E, Yamamoto Y, Casabona A, Persson H, Ibáñez CF. Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. Science. 1995;268:1495–9.
50. Gonzalez M, Ruggiero FP, Chang Q, Shi YJ, Rich MM, Kraner S, Balice-Gordon RJ. Disruption of Trkb-mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. Neuron. 1999;24:567–83.
51. Ip FC, Cheung J, Ip NY. The expression profiles of neurotrophins and their receptors in rat and chicken tissues during development. Neuroscience Letters. 2001;301:107–10.
52. Nagano M, Suzuki H. Quantitative analyses of expression of GDNF and neurotrophins during postnatal development in rat skeletal muscles. Neuroscience Research. 2003;45:391–9.
53. Pitts EV, Potluri S, Hess DM, Balice-Gordon RJ. Neurotrophin and Trk-mediated signaling in the neuromuscular system. International Anesthesiology Clinics. 2006;44:21–76.
54. Garcia N, Santafé MM, Tomàs M, Lanuza MA, Besalduch N, Priego M, Tomàs J. The glial cell line-derived neurotrophic factor (GDNF) does not acutely change acetylcholine release in developing and adult neuromuscular junction. Neuroscience Letters. 2010;480:127–31.
55. Garcia N, Tomàs M, Santafé MM, Besalduch N, Lanuza MA, Tomàs J. The interaction between tropomyosin-related kinase B receptors and presynaptic muscarinic receptors modulates transmitter release in adult rodent motor nerve terminals. The Journal of Neuroscience. 2010;30:16514–22.
56. Je HS, Yang F, Ji Y, Potluri S, Fu X-Q, Luo Z-G, Nagappan G, Chan JP, Hempstead B, Son Y-J, Lu B. ProBDNF and mature BDNF as punishment and reward signals for synapse elimination at mouse neuromuscular junctions. The Journal of Neuroscience. 2013;33:9957–62.
57. Mantilla CB, Zhan W-Z, Sieck GC. Neurotrophins improve neuromuscular transmission in the adult rat diaphragm. Muscle & Nerve. 2004;29:381–6.
58. Amaral MD, Pozzo-Miller L. Intracellular Ca2+ stores and Ca2+ influx are both required for BDNF to rapidly increase quantal vesicular transmitter release. Neural Plasticity. 2012;2012:203536.
59. Belluardo N, Westerblad H, Mudó G, Casabona A, Bruton J, Caniglia G, Pastoris O, Grassi F, Ibáñez CF. Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4. Molecular and Cellular Neurosciences. 2001;18:56–67.
60. Loeb JA, Hmadcha A, Fischbach GD, Land SJ, Zakarian VL. Neuregulin expression at neuromuscular synapses is modulated by synaptic activity and neurotrophic factors. The Journal of Neuroscience. 2002;22:2206–14.
61. Peng HB, Yang J-F, Dai Z, Lee CW, Hung HW, Feng ZH, Ko C-P. Differential effects of neurotrophins and schwann cell-derived signals on neuronal survival/growth and synaptogenesis. The Journal of Neuroscience. 2003;23:5050–60.
62. Colman H, Nabekura J, Lichtman JW. Alterations in synaptic strength preceding axon withdrawal. Science. 1997;275:356–61.
63. Balice-Gordon RJ, Lichtman JW. In vivo observations of pre- and postsynaptic changes during the transition from multiple to single innervation at developing neuromuscular junctions. The Journal of Neuroscience. 1993;13:834–55.
64. Nishizaki T, Sumikawa K. A cAMP-dependent Ca2+ signalling pathway at the endplate provided by the gamma to epsilon subunit switch in ACh receptors. Brain Research Molecular Brain Research. 1994;24:341–5.
65. Lanuza MA, Besalduch N, González C, Santafé MM, Garcia N, Tomàs M, Nelson PG, Tomàs J. Decreased phosphorylation of δ and ε subunits of the acetylcholine receptor coincides with delayed postsynaptic maturation in PKC θ deficient mouse. Experimental Neurology. 2010;225:183–95.
66. Li M-X, Jia M, Yang L-X, Jiang H, Lanuza MA, Gonzalez CM, Nelson PG. The role of the theta isoform of protein kinase C (PKC) in activity-dependent synapse elimination: evidence from the PKC theta knock-out mouse in vivo and in vitro. The Journal of Neuroscience. 2004;24:3762–9.
67. Santafe MM, Priego M, Obis T, Garcia N, Tomàs M, Lanuza MA, Tomàs J. Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse. The European Journal of Neuroscience. 2015;42:1775–87.

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Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process
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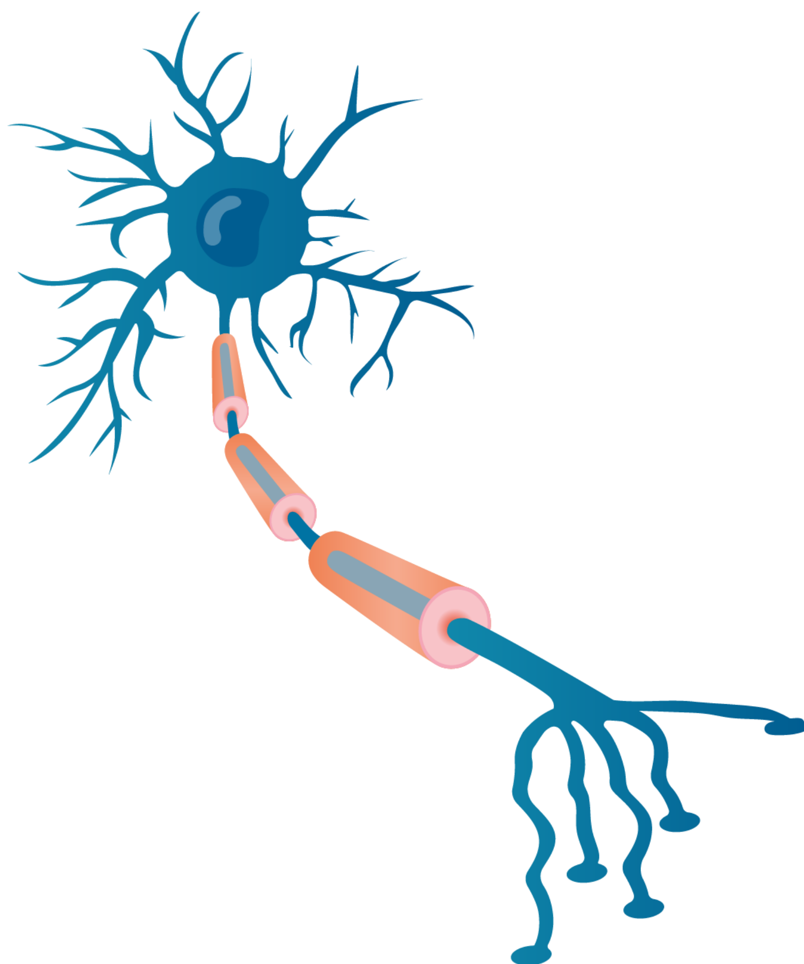

APPENDIX II

UNIVERSITAT ROVIRA I VIRGILI
Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process
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Synergistic Action of Presynaptic Muscarinic Acetylcholine Receptors and Adenosine Receptors in Developmental Axonal Competition at the Neuromuscular Junction

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Keywords

Motor end plate · Motor nerve terminal · Cholinergic synapses · Neuromuscular junction · Presynaptic muscarinic acetylcholine receptors · Adenosine receptors · Synapse elimination

Abstract

The development of the nervous system involves the initial overproduction of synapses, which promotes connectivity. Hebbian competition between axons with different activities leads to the loss of roughly half of the overproduced elements and this refines connectivity. We used quantitative immunohistochemistry to investigate, in the postnatal day 7 (P7) to P9 neuromuscular junctions, the involvement of muscarinic receptors (muscarinic acetylcholine autoreceptors and the M_1, M_2, and M_4 subtypes) and adenosine receptors (A_1 and A_{2A} subtypes) in the control of axonal elimination after the mouse levator auris longus muscle had been exposed to selective antagonists in vivo. In a previous study we analyzed the role of each of the individual receptors. Here we investigate the additive or occlusive effects of their inhibitors and thus the existence of synergistic activity between the receptors. The main results show that the A_{2A}, M_1, M_4, and A_1 receptors (in this order of ability) delayed axonal elimination at P7. M_4 produces some occlusion of the M_1 pathway and some addition to the A_1 pathway, which suggests that they cooperate. M_2 receptors may modulate (by allowing a permissive action) the other receptors, mainly M_4 and A_1. The continued action of these receptors (now including M_2 but not M_4) finally promotes axonal loss at P9. All 4 receptors (M_2, M_1, A_1, and A_{2A}, in this order of ability) are necessary. The M_4 receptor (which in itself does not affect axon loss) seems to modulate the other receptors. We found a synergistic action between the M_1, A_1, and A_{2A} receptors, which show an additive effect, whereas the potent M_2 effect is largely independent of the other receptors (though can be modulated by M_4). At P9, there is a full mutual dependence between the A_1 and A_{2A} receptors in regulating axon loss. In summary, postnatal axonal elimination is a regulated multi-receptor mechanism that involves the cooperation of several muscarinic and adenosine receptor subtypes.

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Introduction

During the development of the nervous system, an initial overproduction of synapses favors connectivity and is followed by an activity-dependent reduction in the number of contacts, which refines and increases the specificity of the neural circuits. Hebbian competition between axons seems to determine this process [1–4]. In newborn animals, skeletal muscle fibers are polyinnervated by several motor axons, but at the end of the axonal competition the end plates are innervated by a single axon [2, 5–12]. Various signaling molecules and presynaptic receptors can play a role in axonal competition by allowing the various nerve endings to have a mutual influence on one another and on the postsynaptic muscle cell and the terminal Schwann cell.

In the neuromuscular junctions (NMJs), several receptors modulate the presynaptic function [13]. Previous results in postnatal muscles indicate the existence of an acetylcholine (ACh) release inhibition mechanism based on muscarinic acetylcholine autoreceptors (mAChRs) coupled to a protein kinase C (PKC) and a voltage-dependent calcium channel (VDCC) intracellular cascade. In some weak motor axons, this mechanism can depress ACh release and even help to disconnect synapses [14–19].

Recently we found that presynaptic mAChRs (M_1, M_2, and M_4 subtypes), adenosine receptors (ARs; A_1 and A_{2A}) and the tropomyosin-related kinase B receptor (TrkB) can cooperate in the developmental synapse elimination process at this synapse [20]. Signaling through these receptors seems to be involved in reducing the initial chance (around postnatal day P7) of eliminating certain weak endings but subsequently increasing (around P9) axonal competition and elimination. However, in spite of the continued presence of the inhibitors of these receptors, monoinnervation is normally achieved at P15. We conclude that the 3 receptor sets intervene in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the losers, although a given axon would finally be eliminated with some autonomy and independently of postsynaptic maturation [20].

In the previous study we analyzed the role of individual receptors. Here we investigate the additive or occlusive effects of the inhibitors of 2 of these receptor sets, mAChRs and ARs (the autoreceptors of ACh and adenosine released by nerve endings), and thus the existence of synergistic activity between them in synapse elimination at the NMJ.

The results indicate that postnatal axonal elimination is a regulated multireceptor mechanism involving the cooperation of several muscarinic and adenosine receptor subtypes. For instance, a synergistic action between M_1, A_1, and A_{2A} receptors promotes axonal loss at P9, whereas the potent axonal loss-promoting effect of M_2 is largely independent of the other receptors.

Material and Methods

Animals

Transgenic B6.Cg-Tg (Thy1-YFP)16 Jrs/J mice were used (The Jackson Laboratory). The mice express spectral variants of GFP (yellow-YFP) at high levels in motor and sensory neurons, and axons are brightly fluorescent all the way to the terminals. All experiments were conducted on Thy1-YFP-expressing mice. In some cases, we checked our results with C57BL/6J mice (The Jackson Laboratory).

Experiments were performed on the levator auris longus (LAL) muscle. Neonatal pups of either sex (7 and 9 days) were obtained and the date of birth was designated P0. We minimized the variability in our measurements by carefully monitoring the timing of conception. Also, the weights of the individuals were within 5% of the mean for a given day after conception. The mice were cared for in accordance with the guidelines of the European Community's Council Directive of November 24, 1986 (86/609/EEC) for the humane treatment of laboratory animals. All experiments on animals were reviewed and approved by the Animal Research Committee of the University Rovira i Virgili (reference No. 0233).

Injection Procedure

The newborn mice were anesthetized with 2% tribromoethanol (0.15 mL/10 g body weight, i.p.). Mice pups received daily subcutaneous injections in the back of the neck beginning on P5 of one or two substances (combinations of 2 selective mAChR antagonists, 2 selective AR antagonists and different combinations of 1 mAChR antagonist plus 1 AR antagonist). Under aseptic conditions, solutions were administered in 50 μL of sterile physiological saline by subcutaneous injection over the LAL external surface as described elsewhere [21]. The animals received 2 or 4 injections from P5, and the LAL muscles were studied on days 7 or 9. Control injections were done in exactly the same way as experimental injections, using phosphate-buffered saline (PBS) by itself. No differences were found between the muscles injected with PBS and those that were not, which suggests that the injection procedure did not in itself induce changes in the overall morphology of the motor end plate and nerve terminals. The solutions were administered at a concentration in accordance with the previously reported biological action of the substance [15, 22, 23]. The final concentration of dimethyl sulfoxide (DMSO) in control and drug-treated preparations was 0.1% (v/v). In specific control experiments, this concentration of DMSO did not affect any of the parameters studied [20].

Tissue Preparation and Histochemistry

Neonatal pups were given a lethal dose of 2% tribromoethanol. Their heads were removed and fixed in 4% paraformaldehyde for

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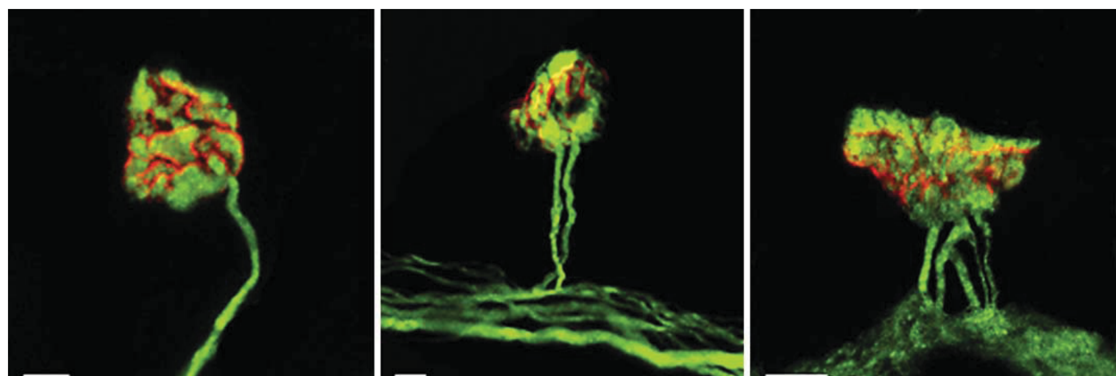

Fig. 1. Confocal fluorescence images. The pictures show singly, dually, and polyinnervated NMJs from P9 YFP mice. Scale bars, 10 μm.

1.5 h. After washing in PBS, the LAL muscles were removed and postfixed for 45 min. After washing in PBS, Thy1-YFP LAL muscles were incubated in PBS containing a 1/800 dilution of 1 μg/mL tetramethylrhodamine-conjugated α-bungarotoxin (TRITC-α-BTX, Molecular Probes, Eugene, OR, USA) for 1 h at room temperature.

Double immunofluorescence and confocal analysis were performed on the C57BL/6J LAL muscle. Whole mounts of LAL were processed to detect the axons with an antibody against a 200-kDa neurofilament protein (rabbit antibody against a 200-kDa neurofilament, 1:1,000; Sigma) and postsynaptic nicotinic ACh receptors with TRITC-α-BTX (Molecular Probes). The appropriate secondary antibody (conjugated with Alexa Fluor 488) donkey anti-rabbit (Molecular Probes) was added and incubated for 4 h. The antibody specificity was tested by incubation in the absence of primary antibody. At least 3 muscles were used as negative controls (not shown). Whole muscles were mounted in Mowiol with p-phenylenediamide (Sigma).

Morphological Analysis and Confocal Microscopy

NMJs were analyzed using an inverted Nikon TE-2000 fluorescent microscope (Nikon, Tokyo, Japan) connected to a personal computer running image analysis software (ACT-1, Nikon). The number of axons per end plate was counted.

Statistical Analysis

All NMJs visible in their entirety were scored, with a minimum of 100 per muscle. At least 6 muscles were studied for each age and condition examined. A Fisher test was applied to compare percentages. The criterion for statistical significance was $p < 0.05$. The categories were scored and the counting was performed by a person with no knowledge of the age or treatment of the animals. The data are presented as means ± SD.

Drugs

Selective A_1R and $A_{2A}R$ Antagonists

The stock solutions were 8-cyclopentyl-1,3-dipropylxanthine (50 mM; DPCPX; C101, Sigma-Aldrich), and 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo(4, 3-e)(1, 2, 4) triazolo(1, 5-c)pyrimidin-5-amine (100 mM; SCH-58261; 2270, Tocris, Bristol, UK), both dissolved in DMSO. Working solutions were DPCPX (100 nM) and SCH-58261 (50 nM).

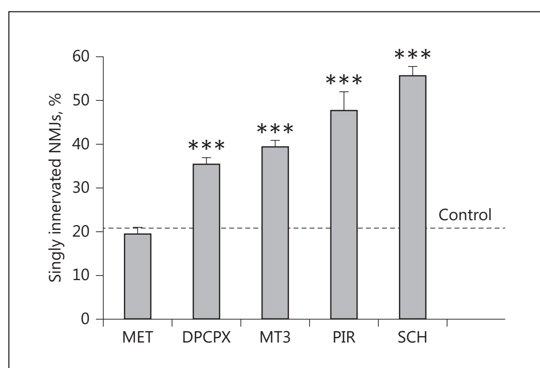

Fig. 2. Individual effect of mAChR and AR inhibitors on axon loss at P7. The figure shows the effect of the inhibitors (1 application each day between P5 and P7) in order of their ability to promote monoinnervation and reduce the percentage of synapses polyinnervated by 2 or more axons. Only the M_2 blocker MET is unable to significantly change the percentage of monoinnervation. The horizontal dotted line marks the control value in muscles injected with PBS. *** $p < 0.05$.

Selective M_1, M_2, and M_4 mAChR Antagonists

The stock solutions were pirenzepine (PIR) dihydrochloride (10 mM; 1071, Tocris Bioscience), methoctramine (MET) (1 mM; M105; Sigma-Aldrich, St. Louis, MO, USA), muscarinic toxin 3 (MT3) (50 μM; M-140; Alomone Labs). The working solutions used were PIR (10 μM), MET (1 μM), and MT3 (100 nM).

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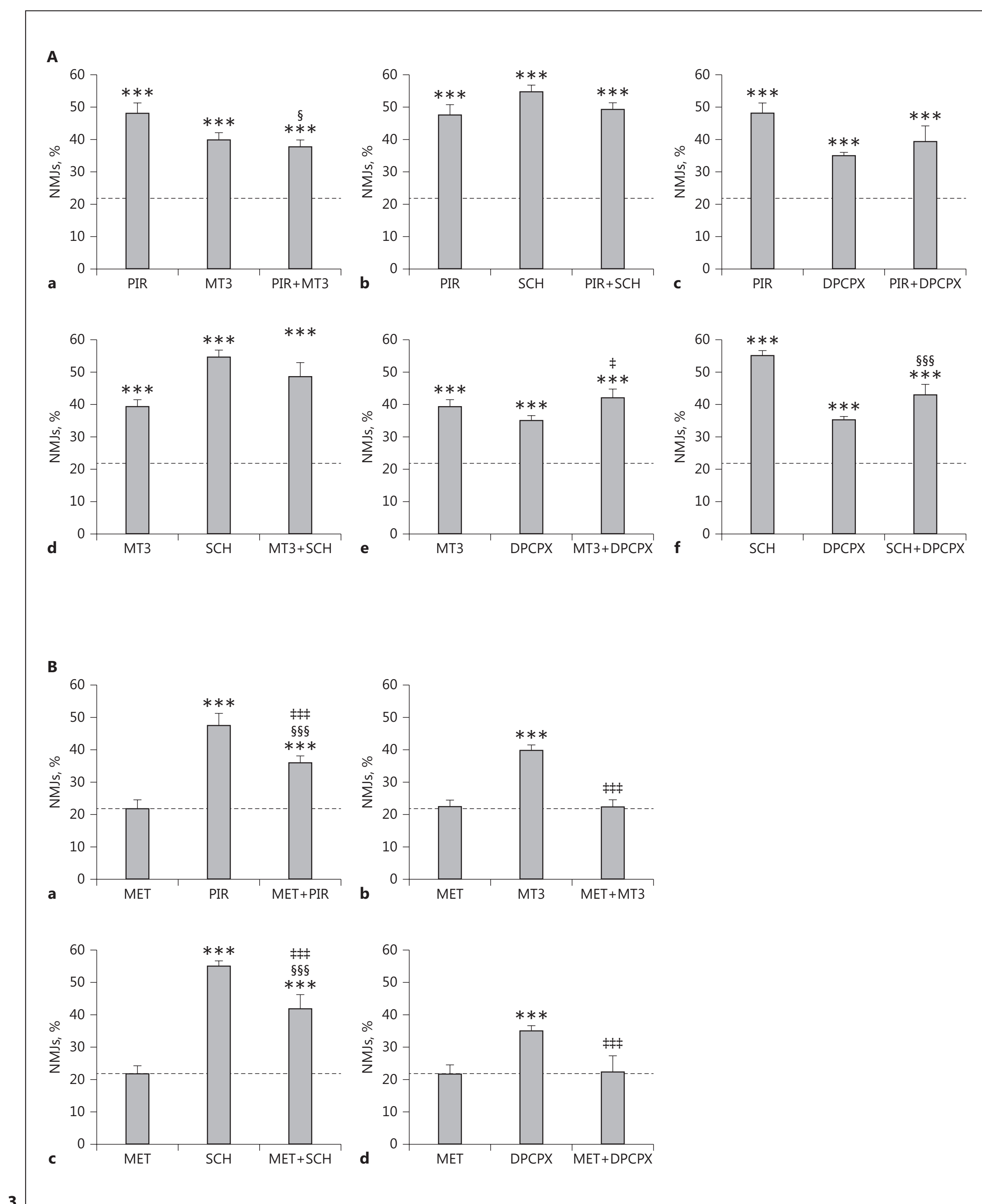

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Results

Individual Role of Presynaptic mAChRs and ARs in Axon Loss Control at P7

Figure 1 shows representative confocal images of the autofluorescent axons in singly, dually, and polyinnervated LAL P9 NMJs from B6.Cg-Tg (Thy1-YFP) mice (hereafter YFP). The role of these presynaptic receptors at P7 was analyzed by using selective inhibitors. The inhibitor action reveals the tonic effect of the receptors in normal conditions. Most of the inhibitors used accelerated transition to monoinnervation in the NMJ and thus accelerated the axonal loss rate. Figure 2 shows that the inhibitor substances (1 application each day between P5 and P7) ranged in their ability to promote monoinnervation and reduce the percentage of synapses that were polyinnervated by 2 or more axons (DPCPX [A_1 subtype inhibitor] < MT3 [M_4 inhibitor] < PIR [M_1 inhibitor] < SCH [SCH-58261 an A_{2A} subtype inhibitor]). Only the M_2 blocker MET was unable to significantly change the percentage of monoinnervation, thus showing that it had no apparent effect on axonal loss. The absolute potency of these various receptors in modulating synapse loss cannot be directly assessed and compared because of the difference in the blocking efficacy of the respective selective inhibitors. However, the relative potency of these substances in accelerating axonal elimination suggests that the corresponding inhibited receptor pathway plays a relatively important role in delaying axonal loss.

Cooperation between mAChRs and ARs at P7

To determine the possible confluence of these muscarinic and purinergic pathways in the control of axonal loss, we investigated the effect of simultaneous incubation with 2 inhibitors (2 antagonists of 2 different receptor subtypes, muscarinic, purinergic, or both) as a pharmacological tool for revealing the possible occlusive or additive crosstalk effects between the corresponding receptors.

To facilitate comparisons, we presented in Figure 3A and B newly reproduced data of some previously published results [20]. The histograms show (dotted line) the percentage of singly innervated NMJs (more single innervation automatically means less multiple innervation) in the untreated B6.Cg-Tg (Thy1-YFP)16 Jrs/J, P7 control mice (2 applications of PBS) and (the bars) after 2 applications (1 application every day after P5) of the mAChR selective antagonists PIR, MET, and MT3, and the selective AR antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo(4, 3-e)(1, 2, 4) triazolo(1, 5-c)pyrimidin-5-amine (SCH-58261, abbreviated here as SCH). Figure 3A (a–f) also shows the percentage of singly innervated NMJs after simultaneous inhibition of 2 mAChRs, a mAChR together with an AR and the 2 ARs together. For the sake of clarity, Figure 3A only shows the associations between the inhibitors that have been proven (Fig. 2) to have an individual effect on axon loss (all but MET). The associations of MET with the other substances are represented in Figure 3B (a–d) and it can be seen that there is an unexpected involvement of the M_2 receptors.

A superficial interpretation of the complex data suggests that blocking 2 different receptors simultaneously (dual associations between PIR, MT3, DPCPX, and SCH) has roughly the same effect on axonal elimination (on the percentage of monoinnervation attained at this time) as blocking only 1 of them. This is true for the associations PIR-SCH, PIR-DPCPX, and MT3-SCH. In these cases, there is no sign that any of these associations have a significantly greater or smaller effect on promoting monoinnervation than the individual effect of the 2 inhibitors. In fact, the final effect is close to the mean value of the 2 substances (e.g., Fig. 3Ad; when MT3 and SCH act independently the mean percentage of monoinnervated junctions is 47.6% ± 1.25 and when they act simultaneously it is 49.32% ± 4.4, Fishers test; n = 1,218 NMJs, n = 4 mice, $p > 0.05$). Thus, for these associations, there is no additive or occlusive effect and the intracellular pathways of the 2 receptors seem to converge in a common mechanism fully activated by the action of only 1 receptor and cannot be increased further or altered by the other receptor. Interestingly, however, when the M_4 blocker MT3 is used in association with the M_1 blocker PIR (Fig. 3Aa) or the A_1

Fig. 3. Cooperation between mAChRs and ARs. **A** Histograms showing the percentage of singly innervated NMJs in the untreated YFP P7 control mice (dotted lines) and after 2 applications (1 application every day after P5) of the mAChR antagonists PIR, MET, and MT3, and the AR antagonist DPCPX and SCH-58261 (SCH). The figure shows also the percentage of singly innervated NMJs after simultaneous inhibition of 2 mAChRs, an mAChR together with an AR and the 2 ARs together. In this figure, for the sake of clarity, we have represented (**a–f**) only associations between the inhibitors proven to have an individual effect on axon loss (all but MET). The associations of MET with the other substances are represented in **B** (**a–d**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ when the correspondent antagonist or combinations of 2 substances is compared with control PBS. § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.005$ when the combination of 2 substances is compared with the first substance. ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.005$ when the combination of 2 substances is compared with the second.

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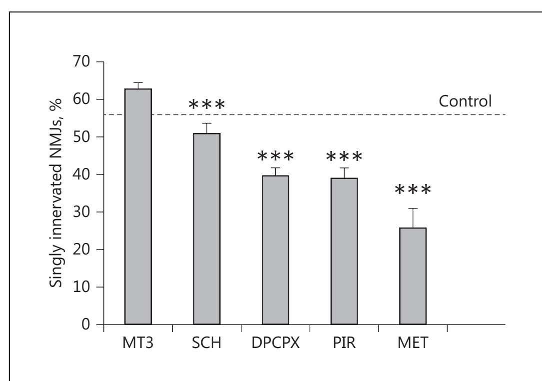

Fig. 4. Individual effect of mAChR and AR inhibitors on axon loss at P9. The figure shows the effect of the selective inhibitors (1 application each day between P5 and P8) in order of their ability to finally delay monoinnervation and keep a high percentage of synapses innervated by 3 or more axons (MET > PIR = DPCPX > SCH-58261). The M_4 blocker MT3 is unable to significantly change the percentage of monoinnervation in this case. The dotted line represents the control value in muscles injected with PBS. *** $p < 0.05$.

blocker DPCPX (Fig. 3Ae), the end result is not significantly different from the individual MT3 effect, but differs significantly from the individual effect of PIR (which is greater) and DPCPX (which is smaller). This may mean that M_4 receptors are more prevalent than M_1 and A_1 receptors. Similarly, when the AR inhibitors DPCPX and SCH act together (Fig. 3Af), the result is no different from when DPCPX acts by itself. However, it is significantly different from the individual effect of SCH, which suggests that A_1 has some sort of permissive effect on the A_{2A} pathway.

As observed in Figure 2, the M_2 mAChR selective blocker MET does not in itself produce any change in the axonal elimination rate during the period considered. Interestingly, however, when the other inhibitors are applied simultaneously with MET, their individual effects are partially or totally occluded (Fig. 3B). First, a partial occlusion of the PIR and SCH effects is observed (Fig. 3Ba and c): the percentage of the monoinnervated NMJ dropped to roughly the mean value between the MET and the substance considered though this value is still different – higher – than that of the untreated control. Second, the presence of MET totally occludes the MT3 and DPCPX effects (Fig. 3Bb and d). Therefore, MET cancels out the effect of the blockers used to prevent the action of M_4 and A_1 (which are the 2 receptors that contribute least to delaying axonal loss). However, the powerful effect of SCH and PIR on axon loss cannot be fully prevented, only lessened, by MET. These data and considerations are represented in Figure 6A in the discussion.

Individual Role of Presynaptic mAChRs and ARs in Axon Loss Control at P9

The receptor inhibitors (1 application each day between P5 and P8) delay transition to monoinnervation in the P9 NMJ. Figure 4 shows the effect of the selective inhibitors in order of their ability to finally delay monoinnervation and keep a high percentage of synapses innervated by 2 or more axons (MET > PIR = DPCPX > SCH). In this case, only the M_4 blocker MT3 is unable to significantly change the percentage of monoinnervation, which shows that there is no apparent effect on axonal loss at this time. Therefore, the 2 receptor sets (mAChRs and ARs) finally promote the conditions of axonal disconnection at the beginning of the second postnatal week (P9) (see also [20]).

Cooperation between mAChRs and ARs at P9

Figure 5 shows the effect of the drug associations applied between P5 and P8 and observed at P9. The data are plotted as they are in Figure 3. The histograms in Figure 5 show the percentage of singly innervated NMJs in the untreated YFP P9 control mice (4 applications of PBS) as a dotted line. Figure 5A (a–f) shows the percentage of NMJs after 4 applications of the mAChR and AR selective antagonists alone and after simultaneous inhibition of the 2 receptors that individually affect axon loss (all the inhibitors but MT3, see Fig. 4). The associations of MT3 with the other substances are represented in Figure 5B (a–d), so that the results are more readily understandable. The data in Figure 5Aa show that the association of the mAChR blockers PIR and MET is not the sum of their individual effects, and the final result is no different from the

Fig. 5. Cooperation between mAChRs and ARs at P9. The data are plotted as they are in Figure 3. **A** Percentage of singly innervated NMJs in controls (PBS, dotted lines) and after exposure (4 applications, 1 application every day after P5) to 1 inhibitor or after simultaneous inhibition of 2 receptors that individually affect axon loss (all inhibitors but MT3, see Fig. 4). The associations of MT3 with the other substances are represented in **B**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ when the correspondent antagonist or combinations of 2 substances is compared with control PBS. § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.005$ when the combination of 2 substances is compared with the first substance. ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.005$ when the combination of 2 substances is compared with the second.

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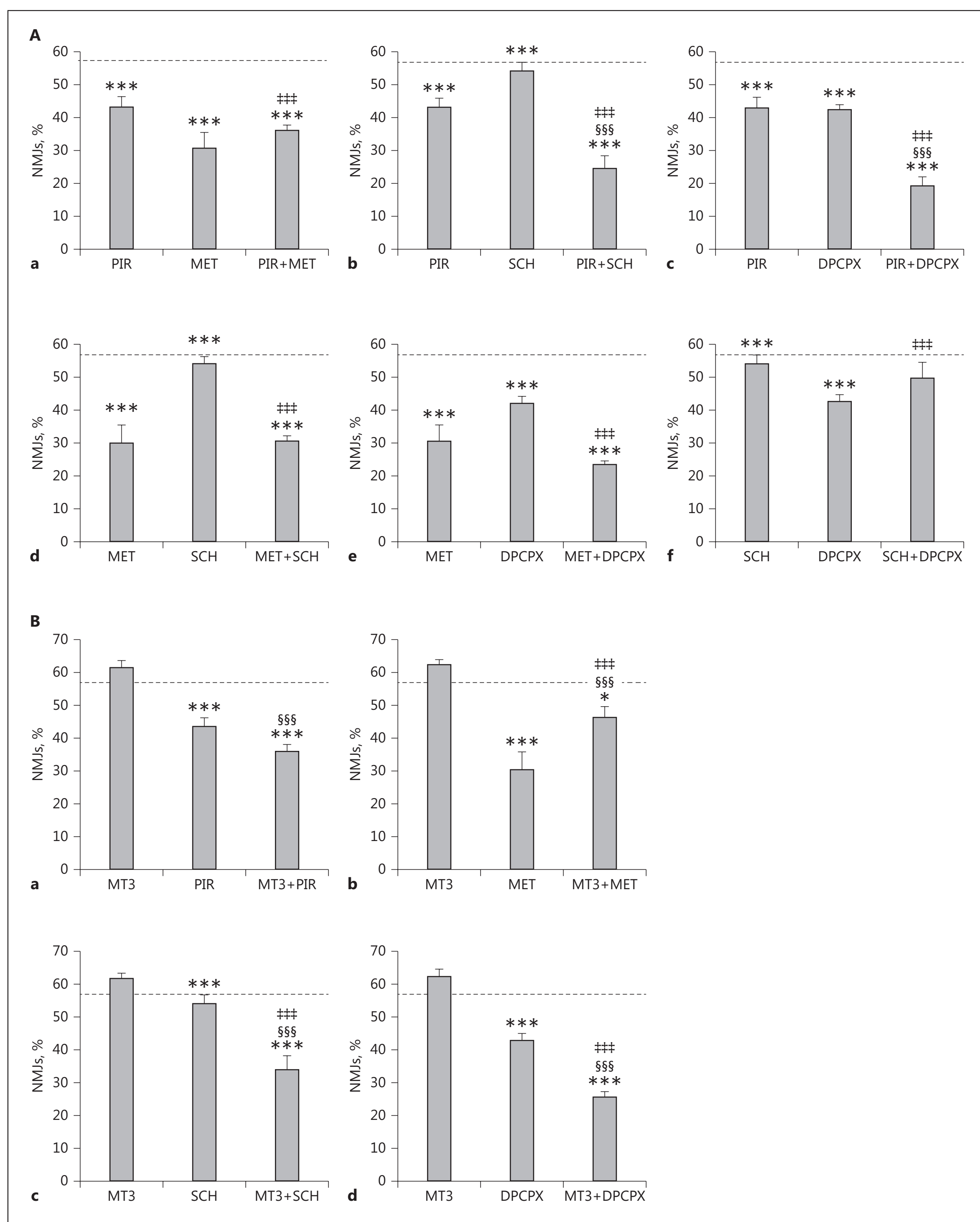

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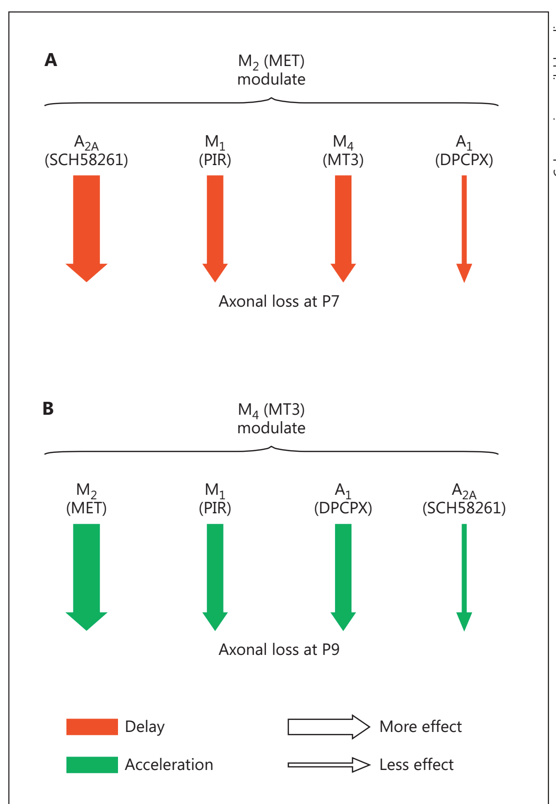

Fig. 6. Diagrams showing an overall representation of the data. **A** P7 muscles; the thickness of the red arrows shows the relative individual ability of the mAChRs and ARs to delay axonal loss at P7 (the thicker they are, the greater their effect; the inhibitor used is noted in brackets near the receptor name). All 4 receptors (A_{2A}, M_1, M_4, and A_1) need to be involved to produce the effect of axonal retention because if any one of them is blocked, then there is no effect and elimination increases. Interestingly, M_4 produces some occlusion of the M_1 and A_1 pathways, which indicates that there is some cooperation between them. Similarly, when the inhibitors of the AR, DPCPX, and SCH act together, the result is no different from the individual DPCPX action. It is, however, significantly different from the individual effect of SCH, which suggests that A_1 has a permissive effect on the A_{2A} pathway. The M_2 receptor does not directly affect axonal elimination because of the lack of effect of MET. However (because MET, in association with the other blockers, partially or totally occludes their individual effects), the M_2 receptors may modulate the other mAChRs and ARs. An alternative explanation is that the M_2 receptors have a more direct and active role and have an axonal elimination-promoting effect. This effect, however, must be lower than the combined elimination preventing-cooperative effect of the A_{2A}, M_1, M_4, and A_1. Therefore, M_2 by itself does not seem to be able to accelerate axon loss, but by blocking only one of the elimination preventing receptors, the axonal retention may be released, thus allowing M_2 to accelerate elimination. **B** P9 muscles; the green arrows with different thicknesses show the relative individual abilities of the mAChR and AR to accelerate axonal loss at P9. The M_1, A_1, and A_{2A} receptors have synergistic effects. Also, A_1 and A_{2A} have a mutual dependence in regulating axon loss at P9. However, the potent effect of the M_2 is not modified by the presence of the M_1, A_1, or A_{2A} blockers. Whereas M_2 modulates the effect of the other receptors at P7, the M_4 receptor (which by itself does not affect axonal elimination) modulates the other receptors at P9. M_4 cooperates positively with M_2 and seems to have a negative influence on A_1 and A_{2A}.

individual effect of PIR. Interestingly, however, both AR inhibitors, DPCPX (Fig. 5Ac) and SCH (Fig. 5Ab), add their own delaying effect on axonal loss to the delaying effect of PIR for an approximate increase of 58 and 36%, respectively. However, the effect of the 2 AR blockers is not added to the effect of MET and the result of the dual drug incubation is no different from the MET effect (Fig. 5Ad and e). When DPCPX and SCH act together (Fig. 5Af), the end result is a percentage of monoinnervation that is no different from that given by the control PBS, which indicates that both pathways are fully occluded.

Figures 4 and 5B show that the M_4 blocker MT3 by itself has no effect on axonal loss at P9. However, Figure 5B shows that the simultaneous application of MT3 with the other muscarinic blockers does not change the effect of PIR (Fig. 5Ba), though it partly occludes the effect of MET which, however, continues to significantly delay axon loss (Fig. 5Bb). Interestingly, MT3 potentiates the delaying effect on axonal loss of both SCH and DPCPX by roughly 32 and 33%, respectively (Fig. 5Bc and d). A representation of these data is shown and discussed in Figure 6B. In some cases, we checked our results with C57BL/6J mice. As an example, in P9 C57BL/J6 animals treated with MT3 plus MET, we found the same result as in YFP animals (In C57BL/J6 animals, [$n = 1{,}075$ NMJs, $n = 3$ mice]: monoinnervated junctions were 42.36 ± 3.54% and in YFP animals they were 48.07 ± 3.54% (Fisher test, $p > 0.05$). No significant differences were observed between YFP and C57BL/6J mice treated with PBS.

Discussion

In addition to the main presynaptic neurotransmitter-postsynaptic receptor interaction within a synapse, several signaling pathways coordinate the pre- and postsyn-

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aptic cells and associated glia. In the NMJ, the final functional outcome of a synaptic contact is the result of metabotropic receptor-mediated signaling acting on the nerve terminal intracellular protein kinases and modulating voltage-dependent channels and the ready releasable pool of synaptic vesicles, which are the instruments of transmitter release [13, 24]. The ability to release ACh is a decisive factor in the stabilization or loss of motor nerve terminals that are in competition to make synapses during development [15, 16, 18, 25–27]. Postsynaptic-derived trophic substances [20] and glial cells [28, 29] also play a decisive role.

The main finding of the present study (which extends a previous finding [20]) is that the coordinated action and cooperation of mAChR (M_1, M_2, and M_4 subtypes) and AR (A_1R and A_{2A}R) signaling modulates developmental axonal competition and affects the axonal loss rate. We used the term "cooperation" to define the collaboration between muscarinic and adenosine receptor pathways in developmental axonal loss control, which requires the receptors to work together and act in conjunction to show an additive or synergistic action or alternatively that their effects are not additive (each receptor can do the job independently of the others). In the present experiments, we simultaneously applied 2 inhibitors (2 antagonists of 2 different receptors) to reveal the possible occlusive or additive crosstalk effects between the corresponding pathways. In our previous experiments [20], we observed 2 well-defined developmental periods: P5–P9 and P9–P15. In the first period, we noticed the complex involvement of these receptors, which finally resulted in promoting axon loss and accelerating monoinnervation of the NMJ. However, in spite of the continued presence of the inhibitors used, the elimination process finished normally at the end of the second week (P15). Our interpretation is that all the receptors intervene initially in modulating the conditions of the competition between nerve endings, possibly helping to determine the winner or the losers, but thereafter the time and conditions of the final elimination occur with some autonomy. Therefore, in the present experiments we focused on the P5–P9 period of clear receptor involvement in axonal competition.

Although it is not clear whether all mAChR subtypes are present in the NMJ [30, 31], some of these receptors play an important regulatory role in ACh release during development [14, 16, 18, 32] and in the adult [16, 33, 34]. In the adult NMJ, M_1 and M_2 mAChRs modulate evoked transmitter release by positive and negative feedback, respectively [14, 31, 34]. However, during NMJ synaptogenesis, the functional significance of the subtypes is different. M_2 receptors promote release in all nerve endings independently of their ACh release level or maturation state, whereas an M_1- and M_4-mediated reduction in release is observed in the weakest endings on polyinnervated dual junctions [14–16, 18, 27, 32]. Similarly, ARs are present in the motor terminals of the newborn and adult NMJs [35, 36]. In the adult, the extent to which inhibitory A_1R and excitatory A_{2A}R modulate the evoked release of ACh [37] seems to depend on the extracellular concentration of adenosine. During development, low concentrations of adenosine may activate both A_1R and A_{2A}R and have a facilitatory action on ACh release [38]. Therefore, mAChRs and ARs (the autoreceptors of the transmitter ACh and the cotransmitter adenosine – derived from ATP hydrolysis – released by the active nerve endings themselves) are good candidates to be involved in postnatal axonal competition and synapse elimination. The involvement of these receptors may allow direct competitive interaction between nerve endings through a differential activity-dependent ACh and adenosine release. So, the more active endings may directly punish the less active endings or reward themselves if the suitable mAChR and AR subtypes are present in the competing axons.

Cooperation between mAChRs and ARs at P7

At P7, about half of the axons are lost from the multi-innervated newborn NMJ [21]. The selective inhibitors of the presynaptic mAChRs (M_1 and M_4 subtypes) and ARs (A_1 and A_{2A}) accelerated axonal elimination when applied on the LAL muscle surface between P5 and P7. This means that the receptors slowed transition to monoinnervation in the untreated NMJ and thus reduced axonal loss (promoted axonal stabilization) when acting individually, at least between P5 and P7 (red arrows in Fig. 6A). Slowing axonal elimination means the temporal retention of some – probably the weakest – axons by increasing their competitive force [20]. By blocking only 1 of these receptors, axonal loss increases (e.g., the 3-to-1 axon transition accelerates). Thus, all 4 receptors are necessary (though with some difference in potency), and axon loss will accelerate if only one is blocked. At least partly for the purpose of synapse elimination, the 4 operators may operate through the same intracellular mechanism. However, the sum of the individual effects of these receptors does not increase axonal retention (simultaneously blocking 2 receptors does not accelerate elimination more than blocking just 1 of them). Interestingly, the effect of blocking M_4 together with M_1 or A_1 is no different from the effect of blocking M_4 by itself, but is significantly different

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from the individual effects of M_1 and A_1. This may mean that M_4 receptors are more prevalent than M_1 and A_1 receptors, which suggests that they may cooperate. Similarly, when AR inhibitors act together, the result is no different than when DPCPX acts by itself. However, it is significantly different from the individual effect of SCH, which suggests that the A_{2A} receptor needs the cooperation of the A_1 pathway for its potent effect. Figure 6A shows an overall representation of the data. The thickness of the red arrows shows the relative individual ability of the mAChRs and ARs to delay axonal loss (the thicker they are, the greater their effect). The links between the corresponding intracellular pathways may determine the delay in axon loss. It seems that all the receptors but M_2 (which does not directly affect axonal elimination because of the lack of effect of MET) are involved in axonal competition. As stated, our results show that all receptors use a common mechanism. These receptors are coupled to intracellular pathways that converge on a limited repertoire of effector kinases to phosphorylate protein targets and materialize structural and functional changes. M_1 and A_1 operate by respectively stimulating and inhibiting the phospholipase C gamma and PKC pathway whereas M_2–M_4 and A_{2A} operate by respectively inhibiting and stimulating the adenyl cyclase (AC) and protein kinase A (PKA) pathway [39–42]. In all cases, however, common final changes such as intracellular calcium oscillations are observed [34, 43]. Interestingly, blocking all PKC isoforms, or the voltage-dependent calcium channels (P/Q-, N- or L-type, or Ca^{2+} influx) or mAChRs (M_1 and/or M_4 subtypes with PIR and MT3, respectively) results in an increase on the size of the synaptic potentials evoked by the weak axons in dual junctions, whereas EPPs evoked by the strongest endings are reduced or unaffected [14–19]. Thus, the difference in ACh release between competing endings is reduced by these muscarinic blockers, and this difference may change the competitive balance of the nerve terminals (at least in a muscarinic-dependent manner). However, as shown here, both PIR and MT3 simultaneously accelerate axon loss at P7, and it is not clear how this acceleration is related to the changes in ACh release (increase in weak endings, decrease in strongest endings) and the presumed decrease in activity-related competition. The peculiar effect of the mAChR on the weakest endings may be related to the specific presence of the M_4 receptor subtype linked to P-, L-, and N-type channels and an inhibitory PKC coupling to ACh release in these contacts [17].

The M_2 subtype is also present in the weak and strong axons in dual junctions [14]. While this receptor links only with P- and N-channels and potentiates ACh release in both nerve endings, we show here that it does not directly affect axonal elimination at P7. However, Figure 6A shows a possible involvement of the M_2 receptors (because the M_2 inhibitor MET, in association with the other blockers, partially or totally occludes their individual effects). A simple explanation is that M_2 receptors modulate by a permissive action the other mAChRs and ARs. An alternative explanation is that the M_2 receptors at P7 have a more direct and active role in concordance with their active role at P9 (see later). This presumed elimination-promoting effect of M_2, however, would be lower than the combined elimination-preventing cooperative effect of A_{2A}, M_1, M_4, and A_1. Therefore, M_2 by itself cannot accelerate elimination, and blocking it with MET does not change axon loss. Blocking only 1 elimination-preventing receptor would release the axonal retention effect and allow M_2 to accelerate elimination. In this context, the observation that blocking A_{2A} or M_1 strongly accelerates axon loss suggests that these 2 receptors neutralize M_2. Because blocking M_2 with MET does not increase axonal retention by itself, the A_{2A}, M_1, M_4, and A_1 ensemble seems to operate at maximum capacity.

Cooperation between mAChRs and ARs at P9

The continued action of these receptors (including the M_2 mAChR subtype but not M_4) between P5 and P9 finally promotes axonal loss and accelerates the monoinnervation of the NMJ (green arrows in Fig. 6B; [20]). This means that the receptors accelerate the transition to monoinnervation and thus promote the destabilization of some axons. All 4 receptors (M_2, M_1, A_1, and A_{2A}) are necessary (though with some variation in potency, which is indicated by the thickness of the green arrows in Fig. 6B) and if only one is blocked, then axon loss is delayed. Also in this period, all receptors converge on a common mechanism. The question at the end of the first postnatal week is why the blockers have such different effects on axon loss between P7 and P9. The receptors' action may shift from delaying axonal loss by favoring the competitive capacities in some nerve endings, to promoting loss between P7 and P9. In fact, one consequence of favoring initial competition around P7 would be an increase in axonal loss at P9. A developmental shift in the functional coupling of some molecules is not such a rare occurrence. Previous studies have shown changes in the role of the mAChRs themselves, the voltage-operated calcium channels (P, N, and L), and PKC during development depending on the maturation level of the NMJ. For instance, PKC couples to potentiate ACh release in the adult NMJ, in the

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strong ending of dual junctions and in the solitary ending in the junctions monoinnervated during maturation. However, as discussed above, their coupling reduces release in the weakest axons in dual junctions and, therefore, any change in the functional expression of PKC may determine the regulation of axonal loss [16, 18].

The experiments clearly show real cooperation between the M_1, A_1, and A_{2A} receptors. We associate one AR blocker (DPCPX or SCH-58261) with the M_1 blocker PIR. We found that both DPCPX and SCH-58261 add their delay on axonal loss to the delaying effect of PIR, 2 increases of roughly 58 and 36%, respectively. These results show an additive or synergistic effect and indicate that at least these receptors cooperate. However, the effect of the M_2 blocker MET is not modified by the presence of the M_1, A_1, or A_{2A} blockers, which indicates that the potent M_2 effect is independent of the other receptors (though, as stated, it seems to be partly modulated by M_4). When DPCPX and SCH act together, the result is a percentage of monoinnervation that is no different from the control PBS, which indicates that both AR pathways are fully dependent on each other and need to cooperate if they are to regulate axon loss.

Whereas M_2 receptors seem to modulate the action of the other mAChRs and ARs at P7, the M_4 receptor (which does not affect synaptic elimination at P9 by itself) can modulate other receptors at this time (Fig. 6B). Specifically, M_4 can cooperate positively with M_2 because MT3 partly occludes the effect of MET. Also, the delaying effect on axonal loss of both DPCPX and SCH-58261 is potentiated (by about 30%) by MT3, which suggests a negative influence of M_4 on A_1 and A_{2A} receptor effects in normal conditions without any inhibitor present.

Relation between mAChRs and ARs

In the adult, ARs and mAChRs heavily depend on each other to modulate ACh release by sharing the PKC and PKA pathways [35, 22]. The influx of external Ca^{2+} required for Ca^{2+}-triggered exocytosis and the fast mode of endocytosis seems to be promoted with the involvement of the presynaptic mAChR [34], and interaction with the AR [44] but also with the BDNF-TrkB receptor pathway [23, 43]. The mAChR and AR pathways (M_1 and A_1 receptors) share a link mediated by the set phospholipase C-phosphatidylinositol 4,5-bisphosphate (PIP2)-diacylglycerol (DAG)-PKC, which modulates P/Q-type voltage-dependent calcium channels [34, 43]. The phospholipase C-generated DAG also regulates the vesicle priming protein Munc13–1 and recruits ACh-containing vesicles for the immediately releasable pool [45]. M_2–M_4 and A_{2A} receptors share the PKA pathway. Previous findings have shown the influence of PKA activity on the coupling of PKC to ACh release and the coordinated involvement of PKC and PKA in the intracellular cascades that modulate neuromuscular transmission [17]. The action of the 2 kinases may be in the same molecules or in different molecules in the release machinery. A protein can be phosphorylated by at least 2 protein kinases, stimulated by different second-messenger systems, which exhibit both overlapping and unique specificities for the phosphorylation of multiple sites in the molecule [46]. The complementary function of these receptors and kinases in the adult reinforces the suggestion that they may have a complementary function in developmental synaptic elimination. We show here that postnatal axonal stabilization or loss is a regulated multireceptor mechanism involving the cooperation of muscarinic (M_1, M_2, and M_4) and adenosine autoreceptor (A_1 and A_{2A}) subtypes in the motor nerve endings. Glia may be implicated as an active participant in these changes. In adults, terminal Schwann cells sense the release of ACh and ATP from the nerve (M_1 and A_1 subtype receptors are present in terminal Schwann cells) and in turn influence transmitter release [47, 48]. In fact, neuregulin 1 signaling between terminal axons and glia during development influences glial cell activation and interposition between the terminal and muscle [29] affecting axon loss. New experiments can be performed to evaluate the downstream mechanism that couples receptors and kinases to the molecular targets responsible of axonal destabilization and retraction.

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Author Contributions

L.N.: data collection, quantitative analysis, literature search, and data interpretation; E.H., A.S., M.T., and V.C.: data collection, literature search; N.G.: quantitative analysis and statistics; J.T., N.G., and M.A.L.: conception and design, literature search, data interpretation, manuscript preparation.

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References

1 Jansen JK, Fladby T: The perinatal reorganization of the innervation of skeletal muscle in mammals. Prog Neurobiol 1990;34:39–90.
2 Sanes JR, Lichtman JW: Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 1999;22:389–442.
3 Purves D, Lichtman JW: Elimination of synapses in the developing nervous system. Science 1980;210:153–157.
4 Chen C, Regehr WG: Developmental remodeling of the retinogeniculate synapse. Neuron 2000;28:955–966.
5 Ribchester RR, Barry JA: Spatial versus consumptive competition at polyneuronally innervated neuromuscular junctions. Exp Physiol 1994;79:465–494.
6 Liu Y, Fields RD, Festoff BW, Nelson PG: Proteolytic action of thrombin is required for electrical activity-dependent synapse reduction. Proc Natl Acad Sci USA 1994;91:10300–10304.
7 Nguyen QT, Lichtman JW: Mechanism of synapse disassembly at the developing neuromuscular junction. Curr Opin Neurobiol 1996;6:104–112.
8 Chang Q, Balice-Gordon RJ: Nip and tuck at the neuromuscular junction: a role for proteases in developmental synapse elimination. Bioessays 1997;19:271–275.
9 Herrera AA, Zeng Y: Activity-dependent switch from synapse formation to synapse elimination during development of neuromuscular junctions. J Neurocytol 2003;32:817–833.
10 Nelson PG, Lanuza MA, Jia M, Li M-X, Tomas J: Phosphorylation reactions in activity-dependent synapse modification at the neuromuscular junction during development. J Neurocytol 2003;32:803–816.
11 Wyatt RM, Balice-Gordon RJ: Activity-dependent elimination of neuromuscular synapses. J Neurocytol 2003;32:777–794.
12 Buffelli M, Busetto G, Bidoia C, Favero M, Cangiano A: Activity-dependent synaptic competition at mammalian neuromuscular junctions. News Physiol Sci 2004;19:85–91.
13 Tomàs J, Santafé MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Obis T, Priego M, Hurtado E: Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. J Neurosci Res 2014;92:543–554.
14 Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J: Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. Eur J Neurosci 2003;17:119–127.
15 Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J: Muscarinic autoreceptors related with calcium channels in the strong and weak inputs at polyinnervated developing rat neuromuscular junctions. Neuroscience 2004;123:61–73.
16 Santafé MM, Lanuza MA, Garcia N, Tomàs M, Tomàs J: Coupling of presynaptic muscarinic autoreceptors to serine kinases in low and high release conditions on the rat motor nerve terminal. Neuroscience 2007;148:432–440.
17 Santafé MM, Garcia N, Lanuza MA, Tomàs M, Tomàs J: Interaction between protein kinase C and protein kinase A can modulate transmitter release at the rat neuromuscular synapse. J Neurosci Res 2009;87:683–690.
18 Santafe MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Tomàs J: Presynaptic muscarinic receptors, calcium channels, and protein kinase C modulate the functional disconnection of weak inputs at polyinnervated neonatal neuromuscular synapses. J Neurosci Res 2009;87:1195–1206.
19 Tomàs J, Santafé MM, Lanuza MA, García N, Besalduch N, Tomàs M: Silent synapses in neuromuscular junction development. J Neurosci Res 2011;89:3–12.
20 Nadal L, Garcia N, Hurtado E, Simó A, Tomàs M, Lanuza MA, Santafé M, Tomàs J: Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes), adenosine receptors (A_1 and A_{2A}) and tropomyosin-related kinase B receptor (TrkB) modulate the developmental synapse elimination process at the neuromuscular junction. Mol Brain 2016;9:67.
21 Lanuza MA, Garcia N, Santafe M, Nelson PG, Fenoll-Brunet MR, Tomas J: Pertussis toxin-sensitive G-protein and protein kinase C activity are involved in normal synapse elimination in the neonatal rat muscle. J Neurosci Res 2001;63:330–340.
22 Santafe MM, Priego M, Obis T, Garcia N, Tomàs M, Lanuza MA, Tomàs J: Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse. Eur J Neurosci 2015;42:1775–1787.
23 Garcia N, Tomàs M, Santafé MM, Besalduch N, Lanuza MA, Tomàs J: The interaction between tropomyosin-related kinase B receptors and presynaptic muscarinic receptors modulates transmitter release in adult rodent motor nerve terminals. J Neurosci 2010;30:16514–16522.
24 Takamori M: Structure of the neuromuscular junction: function and cooperative mechanisms in the synapse. Ann NY Acad Sci 2012;1274:14–23.
25 Colman H, Nabekura J, Lichtman JW: Alterations in synaptic strength preceding axon withdrawal. Science 1997;275:356–361.
26 Buffelli M, Burgess RW, Feng G, Lobe CG, Lichtman JW, Sanes JR: Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. Nature 2003;424:430–434.
27 Santafé MM, Garcia N, Lanuza MA, Uchitel OD, Salon I, Tomàs J: Decreased calcium influx into the neonatal rat motor nerve terminals can recruit additional neuromuscular junctions during the synapse elimination period. Neuroscience 2002;110:147–154.
28 Yang J, Yang H, Liu Y, Li X, Qin L, Lou H, Duan S, Wang H: Astrocytes contribute to synapse elimination via type 2 inositol 1,4,5-trisphosphate receptor-dependent release of ATP. Elife 2016;5:e15043.
29 Lee Y Il, Li Y, Mikesh M, Smith I, Nave K-A, Schwab MH, Thompson WJ: Neuregulin1 displayed on motor axons regulates terminal Schwann cell-mediated synapse elimination at developing neuromuscular junctions. Proc Natl Acad Sci USA 2016;113:479–487.
30 Garcia N, Santafé MM, Salon I, Lanuza MA, Tomàs J: Expression of muscarinic acetylcholine receptors (M_1-, M_2-, M_3- and M_4-type) in the neuromuscular junction of the newborn and adult rat. Histol Histopathol 2005;20:733–743.
31 Wright MC, Potluri S, Wang X, Dentcheva E, Gautam D, Tessler A, Wess J, Rich MM, Son Y-J: Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses. J Neurosci 2009;29:14942–14955.
32 Santafé MM, Garcia N, Lanuza MA, Uchitel OD, Tomás J: Calcium channels coupled to neurotransmitter release at dually innervated neuromuscular junctions in the newborn rat. Neuroscience 2001;102:697–708.
33 Santafé MM, Lanuza MA, Garcia N, Tomàs J: Calcium inflow-dependent protein kinase C activity is involved in the modulation of transmitter release in the neuromuscular junction of the adult rat. Synapse 2005;57:76–84.
34 Santafé MM, Lanuza MA, Garcia N, Tomàs J: Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. Eur J Neurosci 2006;23:2048–2056.
35 Garcia N, Priego M, Obis T, Santafe MM, Tomàs M, Besalduch N, Lanuza MA, Tomàs J: Adenosine A_1 and A_{2A} receptor-mediated modulation of acetylcholine release in the mice neuromuscular junction. Eur J Neurosci 2013;38:2229–22241.
36 Garcia N, Priego M, Hurtado E, Obis T, Santafe MM, Tomàs M, Lanuza MA, Tomàs J: Adenosine A_2B and A_3 receptor location at the mouse neuromuscular junction. J Anat 2014;225:109–117.
37 Correia-de-Sá P, Sebastião AM, Ribeiro JA: Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. Br J Pharmacol 1991;103:1614–1620.

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38 Pousinha PA, Correia AM, Sebastião AM, Ribeiro JA: Predominance of adenosine excitatory over inhibitory effects on transmission at the neuromuscular junction of infant rats. J Pharmacol Exp Ther 2010;332:153–163.
39 Caulfield MP, Birdsall NJ: International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998;50:279–290.
40 Caulfield MP: Muscarinic receptors – characterization, coupling and function. Pharmacol Ther 1993;58:319–379.
41 Felder CC: Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J 1995;9:619–625.
42 Nathanson NM: A multiplicity of muscarinic mechanisms: enough signaling pathways to take your breath away. Proc Natl Acad Sci USA 2000;97:6245–6247.
43 Amaral MD, Pozzo-Miller L: Intracellular Ca^{2+} stores and Ca^{2+} influx are both required for BDNF to rapidly increase quantal vesicular transmitter release. Neural Plast 2012; 2012:203536.
44 Oliveira L, Timóteo MA C-SP: Negative crosstalk between M_1 and M_2 muscarinic autoreceptors involves endogenous adenosine activating A_1 receptors at the rat motor endplate. Neurosci Lett 2009;14:127–131.
45 Bauer CS, Woolley RJ, Teschemacher AG, Seward EP: Potentiation of exocytosis by phospholipase C-coupled G-protein-coupled receptors requires the priming protein Munc13-1. J Neurosci 2007;27:212–219.
46 Chambers TC, Pohl J, Glass DB, Kuo JF: Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. Biochem J 1994; 299:309–315.
47 Jahromi BS, Robitaille R, Charlton MP: Transmitter release increases intracellular calcium in perisynaptic Schwann cells in situ. Neuron 1992;8:1069–1077.
48 Robitaille R: Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. Neuron 1998; 21:847–855.

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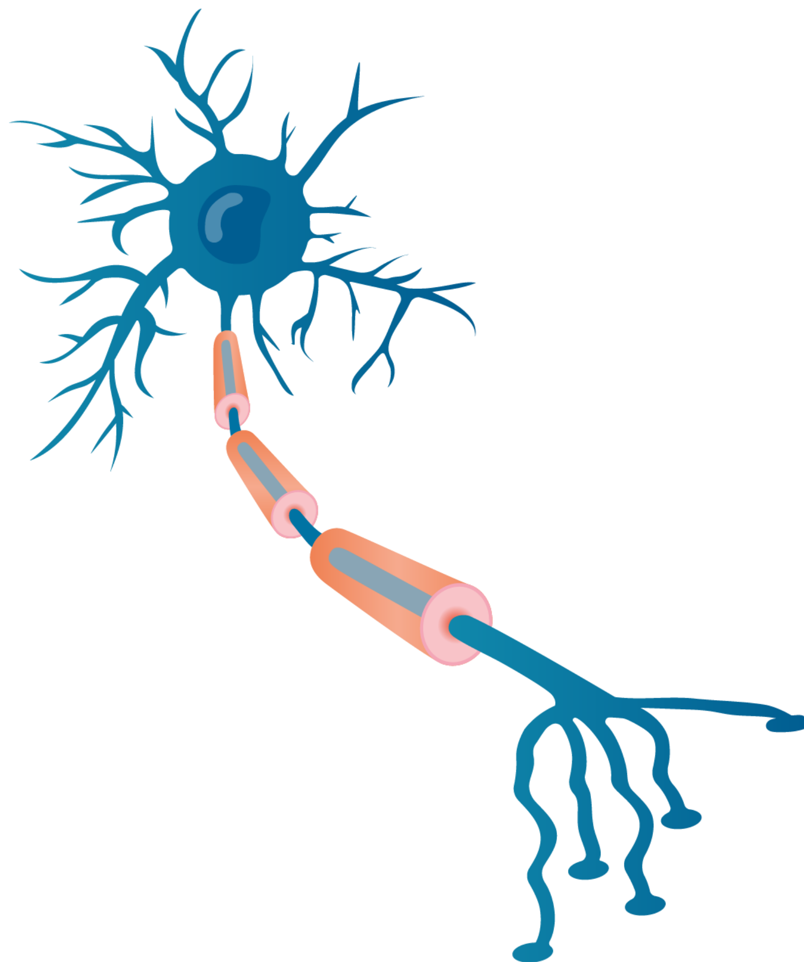

APPENDIX III

UNIVERSITAT ROVIRA I VIRGILI
Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular developmental synapse elimination process
Laura Nadal Magriñà

frontiers in Aging Neuroscience

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Presynaptic Muscarinic Acetylcholine Receptors and TrkB Receptor Cooperate in the Elimination of Redundant Motor Nerve Terminals during Development

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The development of the nervous system involves the overproduction of synapses but connectivity is refined by Hebbian activity-dependent axonal competition. The newborn skeletal muscle fibers are polyinnervated but, at the end of the competition process, some days later, become innervated by a single axon. We used quantitative confocal imaging of the autofluorescent axons from transgenic B6.Cg-Tg (Thy1-YFP)16 Jrs/J mice to investigate the possible cooperation of the muscarinic autoreceptors (mAChR, M_1-, M_2- and M_4-subtypes) and the tyrosine kinase B (TrkB) receptor in the control of axonal elimination after the mice *Levator auris longus (LAL)* muscle had been exposed to several selective antagonist of the corresponding receptor pathways *in vivo*. Our previous results show that M_1, M_2 and TrkB signaling individually increase axonal loss rate around P9. Here we show that although the M_1 and TrkB receptors cooperate and add their respective individual effects to increase axonal elimination rate even more, the effect of the M_2 receptor is largely independent of both M_1 and TrkB receptors. Thus both, cooperative and non-cooperative signaling mechanisms contribute to developmental synapse elimination.

Keywords: motor nerve terminal, cholinergic synapses, neuromuscular junction, presynaptic muscarinic acetylcholine receptors, TrkB receptor, synapse elimination

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INTRODUCTION

During the development of the nervous system, synapses are eliminated on a broad scale (Thompson, 1985; Bourgeois and Rakic, 1993). This allows connectivity to be refined on the basis of Hebbian activity-dependent axonal competition (Jansen and Fladby, 1990; Sanes and Lichtman, 1999). In newborn animals, the skeletal muscle fibers are polyinnervated in the neuromuscular

Abbreviations: AC, adenyl cyclase; ACh, acetylcholine; AR, adenosine receptors; BDNF, Brain-derived neurotrophic factor; EPP, evoke endplate potentials; IP3, inositol triphosphate; LAL, Levator auris longus muscle; mAChR, muscarinic acetylcholine receptor; M_1, M_1-type muscarinic acetylcholine receptor; M_2, M_2-type muscarinic acetylcholine receptor; M_4, M_4-type muscarinic acetylcholine receptor; MET, methoctramine; MT-3, muscarinic toxin 3; nAChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; NT-4, neurotrophin-4; PBS, phosphate buffered saline; PIR, pirenzepine; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TrkB, tropomyosin-related kinase B receptor; VDCC, voltage-dependent calcium channels.

junction area (NMJ; Redfern, 1970; Brown et al., 1976; Ribchester and Barry, 1994), but at the end of the axonal competition, endplates are innervated by a single axon (Benoit and Changeux, 1975; O'Brien et al., 1978; Jansen and Fladby, 1990; Sanes and Lichtman, 1999). This peripheral synapse has been studied extensively as a model for synapse development (Liu et al., 1994; Nguyen and Lichtman, 1996; Chang and Balice-Gordon, 1997; Sanes and Lichtman, 1999; Lanuza et al., 2001; Santafé et al., 2001; Herrera and Zeng, 2003; Wyatt and Balice-Gordon, 2003; Buffelli et al., 2004; Garcia et al., 2010). Several signaling molecules and presynaptic receptors play a role in the axonal competition, which means that the various nerve endings influence one another (Santafé et al., 2009a; Garcia et al., 2010; Nadal et al., 2016). Postsynaptic-derived trophic substances (Nadal et al., 2016) and the participation of glial cells (Lee et al., 2016; Yang et al., 2016) also make a decisive contribution.

In a previous study, we investigated how individual muscarinic acetylcholine receptor (mAChR) subtypes (M_1, M_2 and M_4), adenosine receptors (AR; A_1 and A_{2A}) and tropomyosin-related tyrosine kinase B (TrkB) receptors are involved in the control of synapse elimination in the mouse NMJ (Nadal et al., 2016). The data show that mAChR, AR and TrkB signaling lessen the initial chance of axonal elimination (around P5–P7) by extending the period of axonal competition but then increase (around P9) axonal loss rate (Nadal et al., 2016). The three receptor sets promote axonal disconnection at the beginning of the second postnatal week largely independently of the postsynaptic nicotinic acetylcholine receptor (nAChR) cluster maturation. In addition, a real cooperation between some of the mAChR and AR subtypes is observed. More specifically, preliminary results show that both AR subtypes (A_1 and A_{2A}) can add their independent effect on axonal loss to the effect of the M_1 muscarinic receptor, which leads to greater elimination because of the additive effect of the pathways (Nadal et al., 2016).

In this study, we investigate whether the mAChR subtypes and the TrkB receptor also work together, and whether the respective pathway inhibitors have any additive or occlusive effects and, therefore, whether there is any real cooperation between them in synapse elimination at the NMJ. The main result shows that, like the mAChR and AR relations, the effect of M_1 and TrkB receptors can be added to increase axonal loss rate at P9 but that the effect of M_2 is largely independent of the TrkB receptors. Thus, cooperative and non-cooperative signaling contribute to synapse elimination, which highlights the importance of axonal competition and loss in the development of neural connectivity.

MATERIALS AND METHODS

Animals

Transgenic B6.Cg-Tg (Thy1-YFP)16 Jrs/J mice were used (The Jackson Laboratory,Bar Harbor, ME, USA). The mice express spectral variants of GFP (yellow-YFP) at high levels in motor and sensory neurons, and axons are brightly fluorescent all the way to the terminals.

Experiments were performed on the *Levator auris longus* (LAL) muscle. Neonatal pups of either sex (9 days) were obtained and the date of birth was designated postnatal day 0 (P0). We minimized the variability in our measurements by carefully monitoring the timing of conception. Also, the weights of the individuals were within 5% of the mean for a given day after conception. The mice were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All experiments on animals have been reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 0233).

Injection Procedure

The newborn mice were anesthetized with 2% tribromoethanol (0.15 ml/10 g body weight, i.p.). Mice pups received daily subcutaneous injections in the back of the neck beginning on postnatal day 5 of one or two substances (combinations of two selective mAChR antagonists or of one mAChR antagonist plus the TrkB signaling agent TrkB-Fc). Under aseptic conditions, solutions were administered in 50 μl of sterile physiological saline by subcutaneous injection over the LAL external surface as described elsewhere (Lanuza et al., 2001). The animals received four injections from postnatal day 5, and the LAL muscles were studied on day 9. Control injections were given in exactly the same way as experimental injections, using phosphate buffered saline (PBS) alone. No differences were found between the muscles injected or not with PBS, thus indicating that the injection procedure did not in itself induce changes in the overall morphology of the motor endplate and nerve terminals. The solutions were administered at a concentration in accordance with the previously reported biological action of the substance (Santafé et al., 2004, 2015; Garcia et al., 2010).

Tissue Preparation and Histochemistry

Neonatal pups were given a lethal dose of 2% tribromoethanol. Their heads were removed and fixed in 4% paraformaldehyde for 1.5 h. After washing in PBS, LAL muscles were removed and post-fixed for 45 min. After washing in PBS, Thy1-YFP LAL muscles were incubated in PBS containing a 1/800 dilution of 1 μg/ml tetramethylrhodamine conjugated α-bungarotoxin (α-BTX-TRITC; T1175, Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Whole muscles were mounted in Mowiol with p-phenylenediamide (Sigma).

Confocal Microscopy and Morphological Analysis

NMJs were analyzed using an inverted Nikon TE-2000 fluorescent microscope (Nikon, Tokyo, Japan) connected to a standard personal computer that was running image analysis software (ACT-1, Nikon). The number of axons per endplate was counted. Because of the difficulty of determining the exact number of axonal inputs for each nAChR cluster, when more than two axons converged at the same synaptic site we

classified the NMJs into three groups only: junctions that were monoinnervated, doubly innervated, or innervated by three or more terminal axons. These data enabled us to calculate the "average number of axonal inputs" and the "percentage of polyneuronal innervation" for all fibers receiving two or more axons.

Statistical Analysis

All NMJs visible in their entirety were scored, with a minimum of 100 per muscle. At least six muscles were studied for each age and condition examined. Fisher's test was applied to compare percentages. The criterion for statistical significance was $P < 0.05$. The categories were scored and the counting was performed by a person with no knowledge of the age or treatment of the animals. The data are presented as mean ± SD.

Drugs

Selective M_1, M_2 and M_4 mAChR Antagonists

The stock solutions were pirenzepine (PIR) dihydrochloride (1071, Tocris Bioscience) 10 mM; methoctramine (MET; M105, Sigma—Aldrich, St. Louis, MO, USA) 1 mM; muscarinic toxin 3 (MT3; M-140, Alomone Labs) 50 μM. The working solutions used were PIR (10 μM), MET (1 μM) and MT3 (100 nM).

TrkB Receptor-Related Agent

The following stock solutions were used: recombinant human trkB/Fc Chimera (trkB-Fc; 688-TK; R&D Systems), 100 μg/ml. Working solutions were trkB-Fc 5 μg/ml.

RESULTS

mAChR and TrkB Receptors in Axon Loss Control

Figure 1A shows representative confocal immunofluorescence images of the autofluorescent axons in singly- and polyinnervated LAL P9 NMJs from B6.Cg-Tg (Thy1-YFP) mice (hereafter YFP). When the mAChR subtype-selective inhibitors PIR (M_1 blocker) and MET (M_2 blocker), and the TrkB pathway blocker (a TrkB-Fc chimera) were applied once a day between P5-P8 on the LAL muscle surface, there was a notable delay in the transition to monoinnervation on the NMJ observed at P9 (Nadal et al., 2016). However, the M_4 subtype blocker MT3 shows no effect on axonal loss at this time. **Figure 1B** shows the effect of these selective blockers in increasing order of their relative ability to delay monoinnervation and maintain a high percentage of synapses innervated by three or more axons (PBS-P9 = MT3 < TrkB-Fc < PIR < MET). MET and PIR delayed the three-to-one axon transition whereas TrkB-Fc delayed the two-to-one transition. Therefore, in normal conditions without inhibitors, the two receptor sets (mAChR and TrkB) will contribute to promoting axonal disconnection at the beginning of the second postnatal week (see also Nadal et al., 2016). However, the absolute potency of these receptors in modulating synapse loss cannot be directly compared because the blocking efficacy of the respective selective inhibitors is not the same.

Cooperation Between mAChR and TrkB Receptors

Our experiments were designed to investigate the effect on axonal loss of simultaneous incubation with two inhibitors (two antagonists of two different receptors) and reveal the possible occlusive or additive crosstalk effects between the corresponding pathways. **Figures 1C,D** show the effect of the association of the drugs applied between P5 and P8 and observed at P9. **Figure 1C** shows the percentage of NMJs—of singly-, doubly- and triply- (or more) innervated endplates—after the simultaneous inhibition of two receptors that, individually, clearly modulate axon loss (see **Figure 1B**; all except M_4). The associations of the M_4 blocker MT3 (which does not affect axonal elimination by itself) with the other substances has been represented separately in **Figure 1D** so that the data is more understandable. The M_4 subtype is shown to have a complementary role.

The association of the mAChR blockers PIR and MET shows no additive effect or mutual occlusion in relation to axonal loss (**Figure 1C**). However, the association of the M_1 and TrkB pathway inhibitors (PIR plus TrkB-Fc) results in a clear addition of their respective delaying effects on axonal loss. The percentage of the monoinnervated NMJ after simultaneous exposure to both inhibitors is significantly less (25% of single junctions) than after exposure to only PIR (39%) or only TrkB-Fc (43%). Interestingly, however, the individual effect of the TrkB-Fc does not add to the effect of MET and the result of this dual drug incubation is no different from the effect of MET by itself. The delaying effect of MET on axon loss is the most potent observed in the present experiments and is produced independently of the state of TrkB.

Figure 1B shows that the M_4 blocker MT3 by itself has no effect on axonal loss at P9. **Figure 1D** shows that if MT3 is simultaneously applied with the other blockers it reveal some regulatory or complementary role of M_4 on the other receptors. The presence of MT3 does not change the effect of PIR although some occlusion of the potent effect of MET is observed. MET still continues to significantly delay axon loss (the three-to-one transition), however. Interestingly, the presence of MT3 potentiates the delaying effect of TrkB-Fc on axonal loss, which indicates that the respective receptor pathways (M_4 and TrkB) are cooperating.

These data are represented in **Figure 1E**. The green arrows of different thicknesses show how effective these receptors are at accelerating axonal elimination (the thicker they are, the greater their effect; the inhibitor used is in brackets under the name of the receptor). The M_4 receptor by itself does not affect axonal elimination at P9 (black arrow). Interestingly, the association of the M_1 and TrkB pathway blockers results in the addition of their respective delaying effects on axonal loss, which indicates that the corresponding receptors are cooperating (blue bond between these receptor pathways). However, the potent effect of the M_2 cannot be modified with the simultaneous presence of the M_1 or TrkB blockers. It seems that the M_4 receptor, which does not by itself affect axonal elimination, cooperates positively with M_2 (dotted green arrow). Also, M_4 produces some occlusion of the TrkB pathway (red arrow) but does not cooperate with M_1 (dotted black arrow).

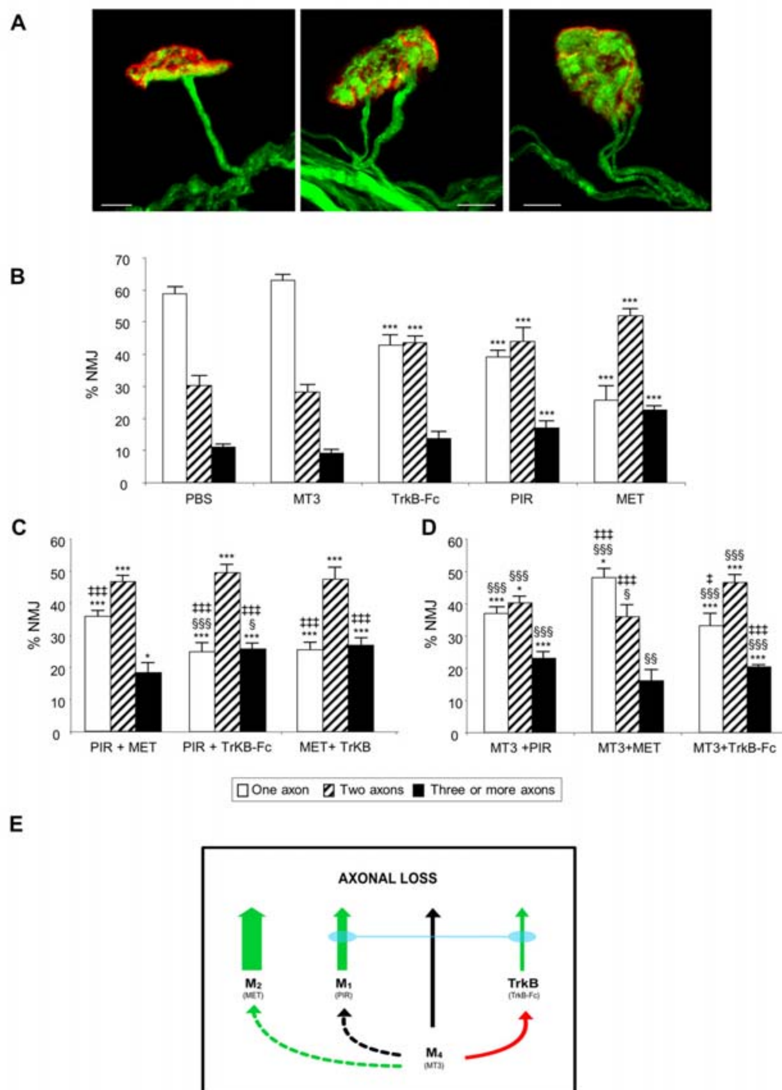

FIGURE 1 | The picture in (A) shows representative confocal immunofluorescence images of singly-, dually- and polyinnervated neuromuscular junction area (NMJ) from P9 YFP mice. Scale bar: 10 μm. The histograms in **(B)** show the percentage of singly-, doubly- and triply- (or more) innervated NMJs in control (phosphate buffered saline, PBS treated) and levator auris longus (LAL) muscles treated with the inhibitors considered. We show newly reproduced data of previous results to facilitate comparison (Nadal et al., 2016). **(C)** shows the percentage of NMJs after the simultaneous inhibition of two receptors clearly involved in axonal elimination (those that affect axon loss rate when they are individually blocked (all except M_4). The associations of the M_4 blocker muscarinic toxin 3 (MT3) with the other inhibitors are represented in **(D)**. An overall representation of the data illustrating the individual role and cooperation of the muscarinic acetylcholine receptor (mAChR) and tyrosine kinase B (TrkB) receptors in developmental axonal loss modulation is shown in the diagram in **(E)**. The green arrows show how effective these receptors are at accelerating axonal elimination (the thicker they are, the greater their effect). The association of the M_1 and TrkB blockers results in the addition of their respective effects (blue bond between these receptors). The M_4 receptor, which does not by itself affect axonal elimination (black arrow), cooperates positively with M_2 (dotted green arrow) and produces some occlusion of the TrkB pathway (red arrow) but does not cooperate with M_1 (dotted black arrow). All NMJs visible in their entirety were scored, with a minimum of 100 synapses per muscle. At least six muscles were studied for each age and condition examined. Fisher's test was applied to compare percentages. When the corresponding antagonist or combinations of two substances were compared with control PBS, significance symbols are: $^{*}P < 0.05$, $^{***}P < 0.005$. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.005$ when the combination of two substances were compared with the first. $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger\ddagger}P < 0.005$ when the combination of two substances were compared with the second.

DISCUSSION

The present experiments show evidence of the cooperation between the presynaptic M_1, M_2 and M_4 mAChR subtypes and the TrkB signaling to modulate the conditions of the developmental axonal competition and loss. In a previous study we found that these receptors (as well as presynaptic AR, A_1 and A_{2A} subtypes) separately contribute to accelerate synapse elimination around P9 in the mouse NMJ (Nadal et al., 2016). It was thought that the muscarinic autoreceptors of the transmitter acetylcholine (ACh) may allow direct competitive interaction between nerve endings through a differential activity-dependent ACh release. The more active axons may directly punish the less active ones or reward themselves (Santafé et al., 2009a). However, an axon that is eliminated at one NMJ may be successful at another (Tomàs et al., 2011), which suggests that other receptors and local postsynaptic- (and glial cell) derived factors are involved. The involvement of the TrkB signaling described may allow a postsynaptic-derived trophic substance such as Brain-derived neurotrophic factor (BDNF) or neurotrophin-4 (NT-4) to make a contribution (Yoshii and Constantine-Paton, 2010).

Interestingly, we observed that both the presynaptic-derived signal (ACh acting on axonal M_1 and M_2 mAChRs) and the TrkB-mediated signal (which may be originated by a postsynaptic-derived NT) have the same effect: namely, the acceleration of supernumerary nerve ending elimination. It seems that the outstanding regulatory resources in the NMJ synaptogenesis are committed to achieving monoinnervation. These presynaptic receptors converge in a common intracellular mechanism and a limited repertoire of effector kinases to phosphorylate protein targets and bring about structural and functional changes leading to axon loss. It is well known that in most cells M_1 and TrkB operate by stimulating the phospholipase C gamma (PLC gamma) and therefore the protein kinase C (PKC) pathway along with the inositol triphosphate (IP3) pathway, whereas M_2–M_4 inhibit the adenyl cyclase (AC) and protein kinase A (PKA) pathway (Caulfield, 1993; Felder, 1995; Caulfield and Birdsall, 1998; Nathanson, 2000). In all cases, however, common final changes such as intracellular calcium oscillations can occur (Santafé et al., 2006; Amaral and Pozzo-Miller, 2012). Both PKA and PKC activity changes have been shown to affect pre- and postsynaptic maturation (Lanuza et al., 2001, 2002). Our present data can be related with the intracellular coupling of the receptors to these serine kinases. Though the blocking efficacy of the selective inhibitors of the muscarinic receptors is not assessed here, M_2 increases the axonal loss rate most with a slight involvement of the M_4 receptor but independently of the M_1 and TrkB receptors. This suggests that downregulation of PKA activity through the couple M_2–M_4 is a key factor in synapse elimination. Concurrently, M_1 and TrkB also contribute separately to axonal loss, but their combined action has a potent summed effect similar to the effect of the M_2 receptor. This suggest that activation of the PLC gamma-PKC pathway through the couple M_1-TrkB may be the other key factor in this process. Thus, a displacement of the PKA/PKC activity ratio to lower values (inhibition of PKA and/or stimulation of PKC) in some nerve endings may have a leading role in synapse elimination. In this context, blockade of PKC in the newborn LAL muscle produces an initial blockade of synapse elimination and a subsequent delay (Lanuza et al., 2002).

In fact, these changes in the kinase activity leading to synapse elimination must occur at least (but not only) in the weakest axons during the competitive interactions. The neurotransmitter release capacity is an important factor in the competing capacity of the various nerve terminals in a NMJ. During development, in the polyinnervated NMJ several nerve endings with different levels of maturation and ACh release capacity get together and compete. The coupling to neurotransmitter release of the considered receptors and kinases is not the same in each of these various endings themselves and in the mature synapses in the adult (Tomàs et al., 2014). So, how does the specific coupling to ACh release of receptors and kinases in the weak and strong axons in competition contribute to axonal loss? As far as serine kinases are concerned, in the adult motor nerve endings both PKA and PKC potentiate ACh release when coupled to neurotransmission (Santafé et al., 2009b). Similarly, the same potentiation is observed in most neuromuscular synapses during development as, for instance, in those formed by the strongest axons (those that evoke the large endplate potential, EPP) in the polyinnervated junctions (Santafé et al., 2004). However, in the weakest endings the inhibition of PKC increases the evoked EPP size indicating that, in normal conditions without any inhibition, this kinase tonically couples to ACh release reduction in these low releasing synapses. Therefore, an M_1-TrkB-mediated increase in PKC activity in the weakest endings would debilitate further their ACh release capacity and competitive force and facilitate their elimination. In addition, an M_2-mediated PKA downregulation in all nerve endings in competition may differentially affect their ACh release and contribute to elimination. Thus, at this point, there is a significant agreement between the known involvement of these molecules in neurotransmission and axon loss.

However, when considering the real postnatal coupling to ACh release of the mAChR and the TrkB receptor in the different nerve endings (the strongest and the weakest) on developing synapses (Santafé et al., 2004; Garcia et al., 2010), additional interpretative keys are needed. In the mature NMJ, M_1 and M_2 subtypes modulate evoked transmitter release by positive and negative feedbacks, respectively (Santafé et al., 2003, 2006). However, during NMJ synaptogenesis, the functional significance of the subtypes is different from in the adult. M_2 receptors promote release in all nerve endings independently of their ACh release level or maturation state whereas an M_1- and M_4-mediated reduction in release is observed in the weakest endings on dual junctions (Santafé et al., 2001, 2002, 2003, 2004, 2007, 2009a). Similarly, the BDNF-TrkB pathway contributes to potentiate ACh release in different neuromuscular adult models but the potentiation is not observed in the weakest nerve endings during development and even some ACh release inhibition was observed in the strongest endings (Garcia et al., 2010). Therefore, interpreting the links and molecular relations between transmitter release and elimination of nerve terminals seems more complex than it seemed at first. The involvement

of other signaling such as AR can contribute to this complexity (Todd and Robitaille, 2006; Nadal et al., 2016). However, some conclusions can be drawn on the basis of all the above data. First, it should be pointed out that, contrary to what happens in the adult, M_1 (and M_4) and PKC activity reduces ACh release in the weakest endings and promotes axonal loss. In fact, blocking mAChRs (M_1- and/or M_4-subtypes) or PKC or voltage-dependent calcium channels (VDCCs; P/Q-, N- or L-type or Ca^{2+} influx) can lead to similar percentage increases in the size of the synaptic potentials evoked by weak axons (Santafé et al., 2003, 2004, 2007, 2009a,b; Tomàs et al., 2011). Therefore, the M_1-PKC pathway may debilitate the ACh release capacity and competitive force of these synaptic contacts and facilitate their elimination. The final target molecules involved may be the VDCC, specially the L-type which is exclusively coupled to ACh release in these weak endings (Santafé et al., 2001) and may contribute to carry high calcium near the molecular mechanism directly involved in axon loss. Second, like M_1 signaling, BDNF-TrkB signaling accelerates axon loss. However, it is not so clear whether it is involved in the modulation of ACh release in the nerve endings that are in competition because it does not affect release in the weak axons. Because PKC effectively reduces release in these endings, the TrkB pathway may operate through the IP3 pathway to increase intracellular calcium and modulate the loss of axons. Third, M_2 promotes axonal elimination the most. However, unlike the adult, this muscarinic subtype promotes ACh release in all the endings that are in competition, including the weakest endings and the solitary ending that finally wins the competition. Therefore, there is a shift in the M_2 coupling during development but how this affects its relation with PKA and how this relates with axonal loss is not known.

AUTHOR CONTRIBUTIONS

LN: data collection, quantitative analysis, literature search and data interpretation; EH, AS and MT: data collection, NG: statistics; JT, NG and MAL: conception and design, literature search, data interpretation, confocal microscopy and manuscript preparation. JT, NG and MAL contributed equally to this work. All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Amaral, M. D., and Pozzo-Miller, L. (2012). Intracellular Ca2+ stores and Ca2+ influx are both required for BDNF to rapidly increase quantal vesicular transmitter release. *Neural Plast.* 2012, 2012–203536. doi: 10.1155/2012/203536

Benoit, P., and Changeux, J. P. (1975). Consequences of tenotomy on the evolution of multiinnervation in developing rat soleus muscle. *Brain Res.* 99, 354–358. doi: 10.1016/0006-8993(75)90036-0

Bourgeois, J. P., and Rakic, P. (1993). Changes of synaptic density in the primary visual cortex of the macaque monkey from fetal to adult stage. *J. Neurosci.* 13, 2801–2820.

Brown, M. C., Jansen, J. K., and Van Essen, D. (1976). Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. *J. Physiol.* 261, 387–422. doi: 10.1113/jphysiol.1976.sp011565

Buffelli, M., Busetto, G., Bidoia, C., Favero, M., and Cangiano, A. (2004). Activity-dependent synaptic competition at mammalian neuromuscular junctions. *News Physiol. Sci.* 19, 85–91. doi: 10.1152/nips.01464.2003

Caulfield, M. P. (1993). Muscarinic receptors–characterization, coupling and function. *Pharmacol. Ther.* 58, 319–379. doi: 10.1016/0163-7258(93)90027-b

Caulfield, M. P., and Birdsall, N. J. (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* 50, 279–290.

Chang, Q., and Balice-Gordon, R. J. (1997). Nip and tuck at the neuromuscular junction: a role for proteases in developmental synapse elimination. *Bioessays* 19, 271–275. doi: 10.1002/bies.950190402

Felder, C. C. (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J.* 9, 619–625.

Garcia, N., Tomàs, M., Santafé, M. M., Besalduch, N., Lanuza, M. A., and Tomàs, J. (2010). The interaction between tropomyosin-related kinase B receptors and presynaptic muscarinic receptors modulates transmitter release in adult rodent motor nerve terminals. *J. Neurosci.* 30, 16514–16522. doi: 10.1523/JNEUROSCI.2676-10.2010

Herrera, A. A., and Zeng, Y. (2003). Activity-dependent switch from synapse formation to synapse elimination during development of neuromuscular junctions. *J. Neurocytol.* 32, 817–833. doi: 10.1023/b:neur.0000020626.29900.fb

Jansen, J. K., and Fladby, T. (1990). The perinatal reorganization of the innervation of skeletal muscle in mammals. *Prog. Neurobiol.* 34, 39–90. doi: 10.1016/0301-0082(90)90025-c

Lanuza, M. A., Garcia, N., Santafé, M., González, C. M., Alonso, I., Nelson, P. G., et al. (2002). Pre- and postsynaptic maturation of the neuromuscular junction during neonatal synapse elimination depends on protein kinase C. *J. Neurosci. Res.* 67, 607–617. doi: 10.1002/jnr.10122

Lanuza, M. A., Garcia, N., Santafé, M., Nelson, P. G., Fenoll-Brunet, M. R., and Tomas, J. (2001). Pertussis toxin-sensitive G-protein and protein kinase C activity are involved in normal synapse elimination in the neonatal rat muscle. *J. Neurosci. Res.* 63, 330–340. doi: 10.1002/1097-4547(20010215)63:4<330::aid-jnr1027>3.0.co;2-w

Lee, Y., Li, Y., Mikesh, M., Smith, I., Nave, K.-A., Schwab, M. H., et al. (2016). Neuregulin1 displayed on motor axons regulates terminal Schwann cell-mediated synapse elimination at developing neuromuscular junctions. *Proc. Natl. Acad. Sci. U S A* 113, E479–E487. doi: 10.1073/pnas.1519156113

Liu, Y., Fields, R. D., Festoff, B. W., and Nelson, P. G. (1994). Proteolytic action of thrombin is required for electrical activity-dependent synapse reduction. *Proc. Natl. Acad. Sci. U S A* 91, 10300–10304. doi: 10.1073/pnas.91.22.10300

Nadal, L., Garcia, N., Hurtado, E., Simó, A., Tomàs, M., Lanuza, M. A., et al. (2016). Presynaptic muscarinic acetylcholine autoreceptors (M1, M2 and M4 subtypes), adenosine receptors (A1 and A2A) and tropomyosin-related kinase B receptor (TrkB) modulate the developmental synapse elimination process at the neuromuscular junction. *Mol. Brain* 9:67. doi: 10.1186/s13041-016-0248-9

Nathanson, N. M. (2000). A multiplicity of muscarinic mechanisms: enough signaling pathways to take your breath away. *Proc. Natl. Acad. Sci. U S A* 97, 6245–6247. doi: 10.1073/pnas.97.12.6245

Nguyen, Q. T., and Lichtman, J. W. (1996). Mechanism of synapse disassembly at the developing neuromuscular junction. *Curr. Opin. Neurobiol.* 6, 104–112. doi: 10.1016/s0959-4388(96)80015-8

O'Brien, R. A., Ostberg, A. J., and Vrbová, G. (1978). Observations on the elimination of polyneuronal innervation in developing mammalian

skeletal muscle. *J. Physiol.* 282, 571–582. doi: 10.1113/jphysiol.1978.sp012482

Redfern, P. A. (1970). Neuromuscular transmission in new-born rats. *J. Physiol.* 209, 701–709. doi: 10.1113/jphysiol.1970.sp009187

Ribchester, R. R., and Barry, J. A. (1994). Spatial versus consumptive competition at polyneuronally innervated neuromuscular junctions. *Exp. Physiol.* 79, 465–494. doi: 10.1113/expphysiol.1994.sp003781

Sanes, J. R., and Lichtman, J. W. (1999). Development of the vertebrate neuromuscular junction. *Annu. Rev. Neurosci.* 22, 389–442. doi: 10.1146/annurev.neuro.22.1.389

Santafé, M. M., Garcia, N., Lanuza, M. A., Tomàs, M., Besalduch, N., and Tomàs, J. (2009a). Presynaptic muscarinic receptors, calcium channels, and protein kinase C modulate the functional disconnection of weak inputs at polyinnervated neonatal neuromuscular synapses. *J. Neurosci. Res.* 87, 1195–1206. doi: 10.1002/jnr.21934

Santafé, M. M., Garcia, N., Lanuza, M. A., Tomàs, M., and Tomàs, J. (2009b). Interaction between protein kinase C and protein kinase A can modulate transmitter release at the rat neuromuscular synapse. *J. Neurosci. Res.* 87, 683–690. doi: 10.1002/jnr.21885

Santafé, M. M., Garcia, N., Lanuza, M. A., Uchitel, O. D., Salon, I., and Tomàs, J. (2002). Decreased calcium influx into the neonatal rat motor nerve terminals can recruit additional neuromuscular junctions during the synapse elimination period. *Neuroscience* 110, 147–154. doi: 10.1016/S0306-4522(01)00543-7

Santafé, M. M., Garcia, N., Lanuza, M. A., Uchitel, O. D., and Tomás, J. (2001). Calcium channels coupled to neurotransmitter release at dually innervated neuromuscular junctions in the newborn rat. *Neuroscience* 102, 697–708. doi: 10.1016/s0306-4522(00)00507-8

Santafé, M. M., Lanuza, M. A., Garcia, N., and Tomàs, J. (2006). Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. *Eur. J. Neurosci.* 23, 2048–2056. doi: 10.1111/j.1460-9568.2006.04753.x

Santafé, M. M., Lanuza, M. A., Garcia, N., Tomàs, M., and Tomàs, J. (2007). Coupling of presynaptic muscarinic autoreceptors to serine kinases in low and high release conditions on the rat motor nerve terminal. *Neuroscience* 148, 432–440. doi: 10.1016/j.neuroscience.2007.06.017

Santafé, M. M., Priego, M., Obis, T., Garcia, N., Tomàs, M., Lanuza, M. A., et al. (2015). Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse. *Eur. J. Neurosci.* 42, 1775–1787. doi: 10.1111/ejn.12922

Santafé, M. M., Salon, I., Garcia, N., Lanuza, M. A., Uchitel, O. D., and Tomàs, J. (2003). Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. *Eur. J. Neurosci.* 17, 119–127. doi: 10.1046/j.1460-9568.2003.02428.x

Santafé, M. M., Salon, I., Garcia, N., Lanuza, M. A., Uchitel, O. D., and Tomàs, J. (2004). Muscarinic autoreceptors related with calcium channels in the strong and weak inputs at polyinnervated developing rat neuromuscular junctions. *Neuroscience* 123, 61–73. doi: 10.1016/j.neuroscience.2003.09.012

Thompson, W. J. (1985). Activity and synapse elimination at the neuromuscular junction. *Cell. Mol. Neurobiol.* 5, 167–182. doi: 10.1007/bf00711091

Todd, K. J., and Robitaille, R. (2006). Purinergic modulation of synaptic signalling at the neuromuscular junction. *Pflugers Arch.* 452, 608–614. doi: 10.1007/s00424-006-0068-3

Tomàs, J., Santafé, M. M., Garcia, N., Lanuza, M. A., Tomàs, M., Besalduch, N., et al. (2014). Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. *J. Neurosci. Res.* 92, 543–554. doi: 10.1002/jnr.23346

Tomàs, J., Santafé, M. M., Lanuza, M. A., García, N., Besalduch, N., and Tomàs, M. (2011). Silent synapses in neuromuscular junction development. *J. Neurosci. Res.* 89, 3–12. doi: 10.1002/jnr.22494

Wyatt, R. M., and Balice-Gordon, R. J. (2003). Activity-dependent elimination of neuromuscular synapses. *J. Neurocytol.* 32, 777–794. doi: 10.1023/b:neur.0000020623.62043.33

Yang, J., Yang, H., Liu, Y., Li, X., Qin, L., Lou, H., et al. (2016). Astrocytes contribute to synapse elimination via type 2 inositol 1,4,5-trisphosphate receptor-dependent release of ATP. *ELife* 5:e15043. doi: 10.7554/eLife.15043

Yoshii, A., and Constantine-Paton, M. (2010). Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity and disease. *Dev. Neurobiol.* 70, 304–322. doi: 10.1002/dneu.20765

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Muscarinic, adenosine and tropomyosin-related kinase B receptor modulate the neuromuscular
developmental synapse elimination process
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