MUSCARINIC RECEPTOR MODULATION OF PROTEIN KINASE A, PROTEIN KINASE C AND EXOCYTOTIC PROTEINS AT THE NEUROMUSCULAR JUNCTION

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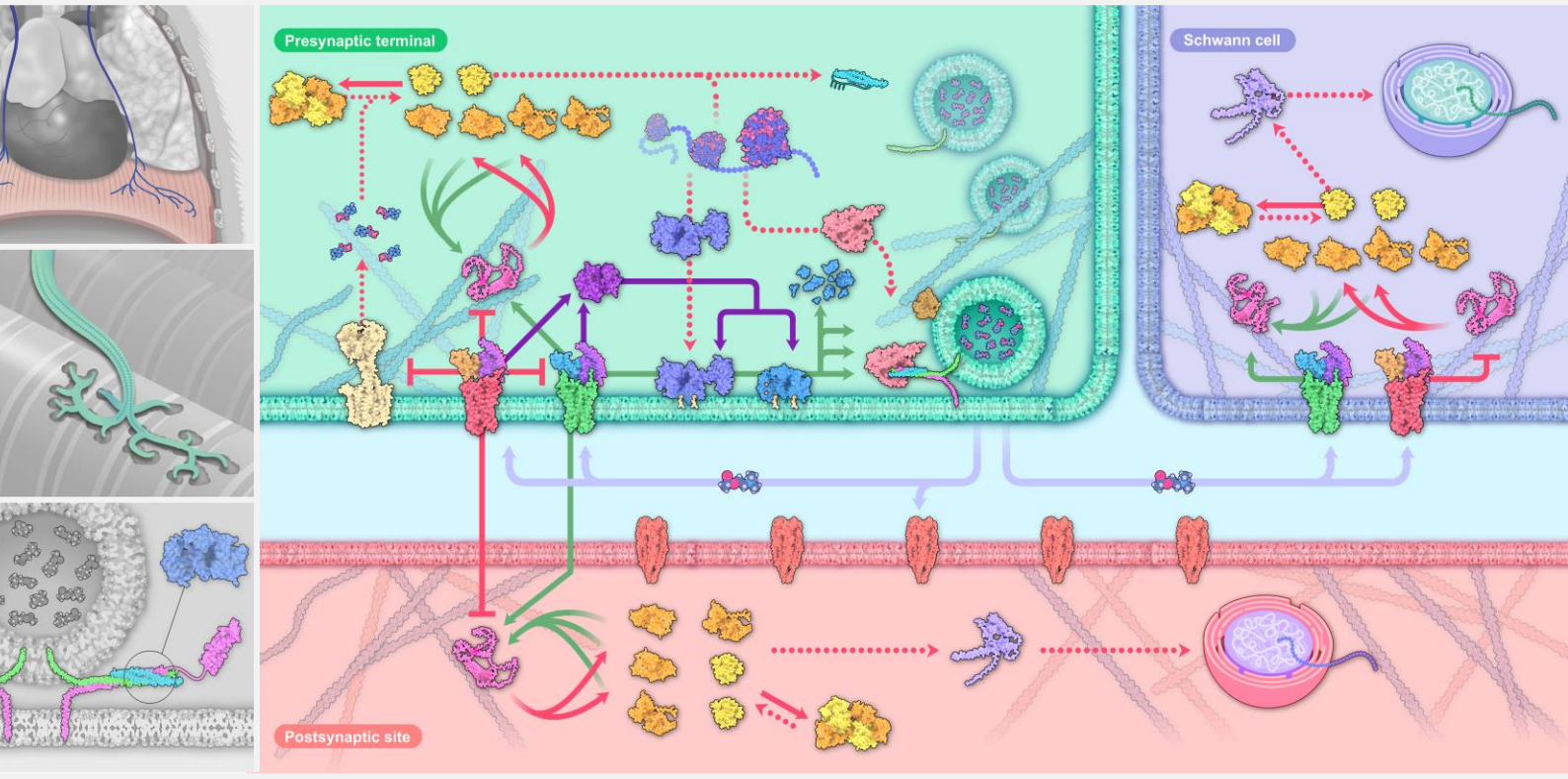
UNIVERSITAT ROVIRA i VIRGILI

Muscarinic receptor modulation of protein kinase A, protein kinase C and exocytotic proteins at the neuromuscular junction

VÍCTOR CILLEROS MAÑÉ



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Doctoral Thesis

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Unitat d'Histologia i Neurobiologia

Departament de Ciències Mèdiques Bàsiques

UNIVERSITAT ROVIRA i VIRGILI

Reus

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UNITAT D'HISTOLOGIA I NEUROBIOLOGIA
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FACULTAT DE MEDICINA I CIÈNCIES DE LA SALUT

FAIG CONSTAR que aquest treball, titulat "Muscarinic receptor modulation of protein kinase A and protein kinase C isoforms and exocytotic proteins at the neuromuscular junction", que presenta Víctor Cilleros-Mañé per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Ciències Mèdiques Bàsiques d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "Muscarinic receptor modulation of protein kinase A and protein kinase C isoforms and exocytotic proteins at the neuromuscular junction", que presenta Víctor Cilleros-Mañé para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Ciencias Médicas Básicas de esta universidad.

I STATE that the present study, entitled "Muscarinic receptor modulation of protein kinase A and protein kinase C isoforms and exocytotic proteins at the neuromuscular junction", presented by Víctor Cilleros-Mañé for the award of the degree of Doctor, has been carried out under my supervision at the Department Basic Medical Sciences of this university.

Reus, 5 de setembre de 2022

Els directors de la tesi doctoral
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Simplicity is the final achievement. After one has played a vast quantity of notes and more notes, it is simplicity that emerges in all its charm as the ultimate crowning reward of art.

—Frederic Chopin. From Madame Streicher's Recollections

– Aprendí... mirando por la ventana. Todos los miércoles cuando ibais a clase, mirando por la ventana y me aprendí todos los pasos. Para enseñárselos a mi madre.

—Paquita Salas

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ABSTRACT

The neuromuscular junction (NMJ) uses plastic mechanisms to adjust the release of acetylcholine (ACh) to an incredibly dynamic environment. Muscarinic acetylcholine receptors (mAChRs) participate as autoreceptors, tuning neurotransmission. The M_1 subtype activates protein kinase C (PKC) to enhance the release, whereas M_2 inhibits protein kinase A (PKA) to decrease it. The captivating research in the past decade has provided extensive electrophysiological knowledge about muscarinic signaling. However, the molecular data accompanying this knowledge was limited; and the role of some second messengers remained elusive. Therefore, the present thesis aimed to characterize how M_1 and M_2 mAChRs regulate the multiple PKA and PKC subunits, their scaffolds and exocytotic targets.

We analyzed the muscarinic cascade at the rat diaphragm muscle by testing selective and general inhibitors of M_1 and M_2 mAChR, nPKCε, cPKCβI, PKA and PDK1 and analyzed each alteration mainly by Western blotting as well as subcellular fractionation and co-immunoprecipitation. We also made use of immunohistochemical and confocal techniques to corroborate the presynaptic location of our molecules of interest.

Our results show that M_1 receptors are downregulated by the M_2 pathway. Regarding PKA signaling, M_2 inhibits PKA activity by downregulating Cβ subunit, upregulating RIIα/β and liberating RIβ and RIIα to the cytosol, which reduces the phosphorylation of SNAP-25 on Thr^{138} and CREB. M_1 signaling crosstalks with M_2/PKA by recruiting R subunits to the membrane. Regarding PKC signaling, both M_1 and M_2 mAChR activate the master kinase PDK1, which promotes the priming of the presynaptic PKCβI and PKCε isoforms. M_1 recruits both primed PKCs to the membrane and promotes Munc18-1, SNAP-25 and MARCKS phosphorylation. In contrast, M_2 downregulates PKCε through a PKA-dependent pathway, which inhibits Munc18-1 synthesis and its PKC-phosphorylation.

The results demonstrate that M_1 and M_2 mAChRs perform a coordinated and interdependent signaling to modulate neurotransmission at the NMJ.

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RESUM

La unió neuromuscular utilitza mecanismes de plasticitat per adequar l'alliberament d'acetilcolina (ACh) dins d'un entorn molt dinàmic. Els receptors muscarínics d'acetilcolina (mAChRs) participen com a autoreceptors, ajustant la neurotransmissió. El subtipus M_1 activa la proteïna quinasa C (PKC) per potenciar la transmissió, mentre que l'M_2 inhibeix la proteïna quinasa A (PKA) per reduir-la. En la passada dècada, grans descobriments ens han aportat un coneixement electrofisiològic extens sobre la senyalització muscarínica. Tanmateix, les dades moleculars segueixen sent escasses i el rol d'alguns segons missatgers resta desconegut. Així doncs, aquesta tesi es realitza amb l'objectiu de caracteritzar com els receptors M_1 i M_2 modulen les isoformes de PKA i PKC, les seves proteïnes reguladores i les dianes d'exocitosi.

Hem analitzat la cascada muscarínica al múscul diafragma de rata usant inhibidors selectius i generals de M_1, M_2, nPKCε, cPKCβI, PKA i PDK1 i analitzant cada alteració mitjançant Western blot, així com fraccionament subcel·lular i co-immunoprecipitació. També hem fet ús de tècniques immunohistoquímiques i microscòpia confocal per corroborar la localització presinàptica de les molècules d'interès.

Els resultats mostren que els nivells del receptor M_1 són disminuïts per la via de l'M_2. Respecte a la senyalització de la PKA, l'M_2 inhibeix la seva activitat regulant la subunitat Cβ a la baixa, la subunitat RIIα/β a l'alta i alliberant RIβ i RIIα al citosol, el que redueix la fosforilació de SNAP-25 (Thr^{138}) i CREB. L'M_1 s'interposa en la senyalització M_2/PKA reincorporant les subunitats R a la membrana. Respecte la senyalització PKC, ambdós M_1 i M_2 poden activar la quinasa mestra PDK1, que promou la maduració de les isoformes de PKC βI i ε presinàptiques. L'M_1 recluta les dues PKC madures a la membrana i promou la fosforilació de Munc18-1, SNAP-25 i MARCKS. Al contrari, l'M_2 regula a la baixa la PKCε de forma dependent de PKA, el que inhibeix la síntesi de Munc18-1 i la seva fosforilació.

El treball present contribueix a comprendre l'acció conjunta i interdependent dels receptors M_1 i M_2 per regular la neurotransmissió.

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RESUMEN

La unión neuromuscular utiliza mecanismos de plasticidad para adecuar la liberación de acetilcolina (ACh) a un entorno muy dinámico. Los receptores muscarínicos de acetilcolina (mAChRs) participan como autorreceptores, ajustando la neurotransmisión. El subtipo M_1 activa la proteína quinasa C (PKC) para potenciar la transmisión, mientras que el M_2 inhibe la proteína quinasa A (PKA) para reducirla. La interesante investigación de la pasada década nos ha aportado un extenso conocimiento electrofisiológico sobre la señalización muscarínica. Aun así, siguen siendo escasos los datos moleculares y el rol de algunos segundos mensajeros permanece desconocido. Así pues, esta tesis tiene el objetivo de caracterizar cómo los receptores M_1 y M_2 regulan las isoformas de PKA y PKC, sus proteínas reguladoras y las dianas de exocitosis.

Para ello, hemos analizado la cascada muscarínica en el músculo diafragma de rata usando inhibidores selectivos y generales de M_1, M_2, nPKCε, cPKCβI, PKA y PDK1 y analizando dichas alteraciones mediante Western blot, fraccionamiento subcel·lular y co-inmunoprecipitación. También hemos hecho uso de técnicas inmunohistoquímicas y confocales para corroborar la localización presináptica de nuestras moléculas de interés.

Los resultados muestran que los niveles del receptor M_1 son disminuidos por la vía del M_2. Respecto a la señalización PKA, M_2 inhibe su actividad total disminuyendo la subunidad Cβ, aumentando las subunidades RIIα/β y liberando a RIβ y RIIα al citosol, lo que reduce la fosforilación de SNAP-25 (Thr^{138}) y CREB. El receptor M_1 se interpone en la señalización M_2/PKA reincorporando las subunidades R a la membrana. Respecto la señalización PKC, ambos M_1 y M_2 pueden activar la quinasa maestra PDK1, que promueve la maduración de las isoformas PKCβI y ε presinápticas. M_1 recluta las dos PKC maduras a la membrana y promueve la fosforilación de Munc18-1, SNAP-25 y MARCKS. Al contrario, el M_2 inhibe la PKCε de forma dependiente de PKA, lo que también disminuye la síntesis de Munc18-1 y su fosforilación. Este trabajo contribuye a comprender la acción conjunta e interdependiente de los receptores M1 y M2 sobre la neurotransmisión.

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Víctor Cilleros Mañé

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ABBREVIATIONS

The complete chemical nomenclature of complex chemical compounds is indicated in the Materials and Methods section.

4-DAMP	1,1-dimethyl-4-diphenylacetoxipiperidinium
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AKAP	A-kinase-anchoring proteins
ATP	Adenosine triphosphate
Atr	Atropine
BDNF	Brain-derived neurotrophic factor
C	PKA catalytic subunit
Cα	PKA catalytic subunit α
Cβ	PKA catalytic subunit β
Cγ	PKA catalytic subunit γ
Ca^{2+}	Calcium ion
CaC	Calphostin C
cAMP	3',5'-cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
CNS	Central nervous system
COP I	Coat complex protein I
cPKC	Conventional protein kinase C
CRE	cAMP-response element

CREB	CRE-binding protein
DAG	Diacylglycerol
EDL	*Extensor digitorum longus* muscle
EPP	Evoked endplate potential
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor (GPCR) kinase
Hsp90	Heat shock protein 90
Hz	Hertz
IHC	Immunohistochemistry
IP_3	Inositol trisphosphate
kDa	Kilodalton
LAL	*Levator auris longus*
LTP	Long-term potentiation
$M_{1\text{-}5}$	Muscarinic acetylcholine receptor subtype 1-5
mAChR	Muscarinic acetylcholine receptor
MARCKS	Myristoylated Alanine-Rich protein Kinase C Substrate
MEPP	Miniature endplate potential
Met	Methoctramine
Mg^{2+}	Magnesium ion
Munc18-1	Mammalian homologue of Uncoordinated-18
nAChR	Nicotinic acetylcholine receptors
NMJ	Neuromuscular junction
NSF	N-ethylmaleimide-sensitive factor
PDE	Phosphodiesterase
PDK1	3-phosphoinositide-dependent kinase 1

PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
Pir	Pirenzepine
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
mRNA	messenger ribonucleic acid
PS	Pseudosubstrate region
PTX	Pertussis toxin
PVDF	Polyvinylidene difluoride
R	PKA regulatory subunit
RIα	PKA regulatory subunit Iα
RIβ	PKA regulatory subunit Iβ
RIIα	PKA regulatory subunit IIα
RIIβ	PKA regulatory subunit IIβ
RACK	Receptor for activated C-kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
SM protein	Sec1/Munc18-like proteins
SNAP	Soluble NSF Attachment Protein
SNAP-25	Synaptosomal-associated protein of 25 kDa
SNAP-25B	Isoform B of SNAP-25
SNARE	Soluble NSF Attachment Protein Receptor
Thr	Threonine

TMD	Transmembrane domain
TRICT	Tetramethylrhodamine
TrkB	Tropomyosin related kinase B
VAMP	Vesicle-associated membrane protein
VGCC	Voltage-gated calcium channels
VGSC	Voltage-gated sodium channels
WB	Western blot
αBTX	α-bungarotoxin
βIV_{5-3}	PKCβI translocation inhibitory peptide
εV_{1-2}	PKCε translocation inhibitory peptide
µg	Microgram (10^{-6} g)
µl	Microliter (10^{-6} l)
µm	Micrometer (10^{-6} m)
µM	Micromolar (10^{-6} M)

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CHAPTER 1

INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
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CHAPTER 1

INTRODUCTION

Structural Outline

*In **Chapter One – Introduction**, the context of the thesis is introduced in five sections. Section 1 provides the context of the neuromuscular junction and the molecules of interest in this thesis. Sections 2–4 review the existing literature on muscarinic receptors and their downstream cascades. The research objectives and questions have been identified.*

1. The neuromuscular junction

The specialized interface between the nervous system and the skeletal muscle is the **neuromuscular junction** (NMJ), also known as neuromuscular synapse or motor endplate because of its platter shape. This chemical synapse controls the excitation and mechanical response of the skeletal muscle, which generates physical movement. NMJs are classified as nicotinic cholinergic synapses because **acetylcholine** (ACh) is the major neurotransmitter operating at the skeletal neuromuscular junction of vertebrates.

To coordinate body movement, skeletal muscle fibers (also called myocytes) are innervated by α motoneurons. The soma of these neurons rests in the central nervous system (CNS), specifically, in the ventral horn of the spinal cord. Their axon usually reaches long distances from the CNS through the peripheral nerves until it arrives to the target muscle. Once inside the muscle, their axon branches off in slight processes, the **nerve terminals**, only innervating myocytes of a single muscle organ. One motoneuron and all the muscle fibers that it innervates (in some cases up to one hundred) comprise a motor unit. Even though a single neuron innervates various myocytes, each adult myocyte is only innervated by one single neuron.

1.1. The three cellular components

The NMJ is comprised of three cellular components (■ Figure 1-A). The terminal ending of a motoneuron is the **presynaptic component**, the muscle fiber area underneath the synaptic contact is the **postsynaptic component** and Schwann cells are the **perisynaptic component**. The three cells form a tripartite synapse, working together to activate, respond and regulate neurotransmission. The space between the pre- and postsynaptic components and enclosed by the terminal Schwann cells is known as the synaptic cleft. All three cells are highly specialized at the most proximal site to the NMJ, having organelles and molecules scarce or non-existent outside de synaptic zone.

The alpha motoneuron

Motoneurons are cells that convey commands coded as electrical action potentials from the CNS to a muscle, a gland or another effector organ. Structurally, motoneurons are comprised by a cell body, the soma; dendrites and an axon, which are cellular processes extending from the soma. The cell body is located at the ventral horn of the spinal cord and receives information from the CNS through interneurons. Axons extend long distances from the soma and ramify intramuscularly, contacting hundreds of muscle fibers to form a motor unit. The most distal part of these axonal projections is called the **nerve terminal**, a complex structure of even slender projections specialized in neurotransmitter release through exocytosis (■ Figure 1-B).

Organelles like the nuclei, endoplasmic reticulum, Golgi apparatus, or lysosomes are confined in the soma of motoneurons. In contrast, mitochondria are distributed both in the soma and along the axon, being especially abundant in terminal boutons to supply the required energy for the vesicle cycle, among other mechanisms. Ribosomes and mRNA pools are also present in the synaptic terminal to quickly respond to demands of specific synaptic proteins. The neuronal axon is rich in cytoskeletal fibers microfilaments, microtubules and neurofilaments to maintain its architecture and enhance cellular processes like vesicle transport. Neurofilaments are a class of intermediate filaments that provide axonal structural support and microtubules provide structural support and a platform for cell transport.

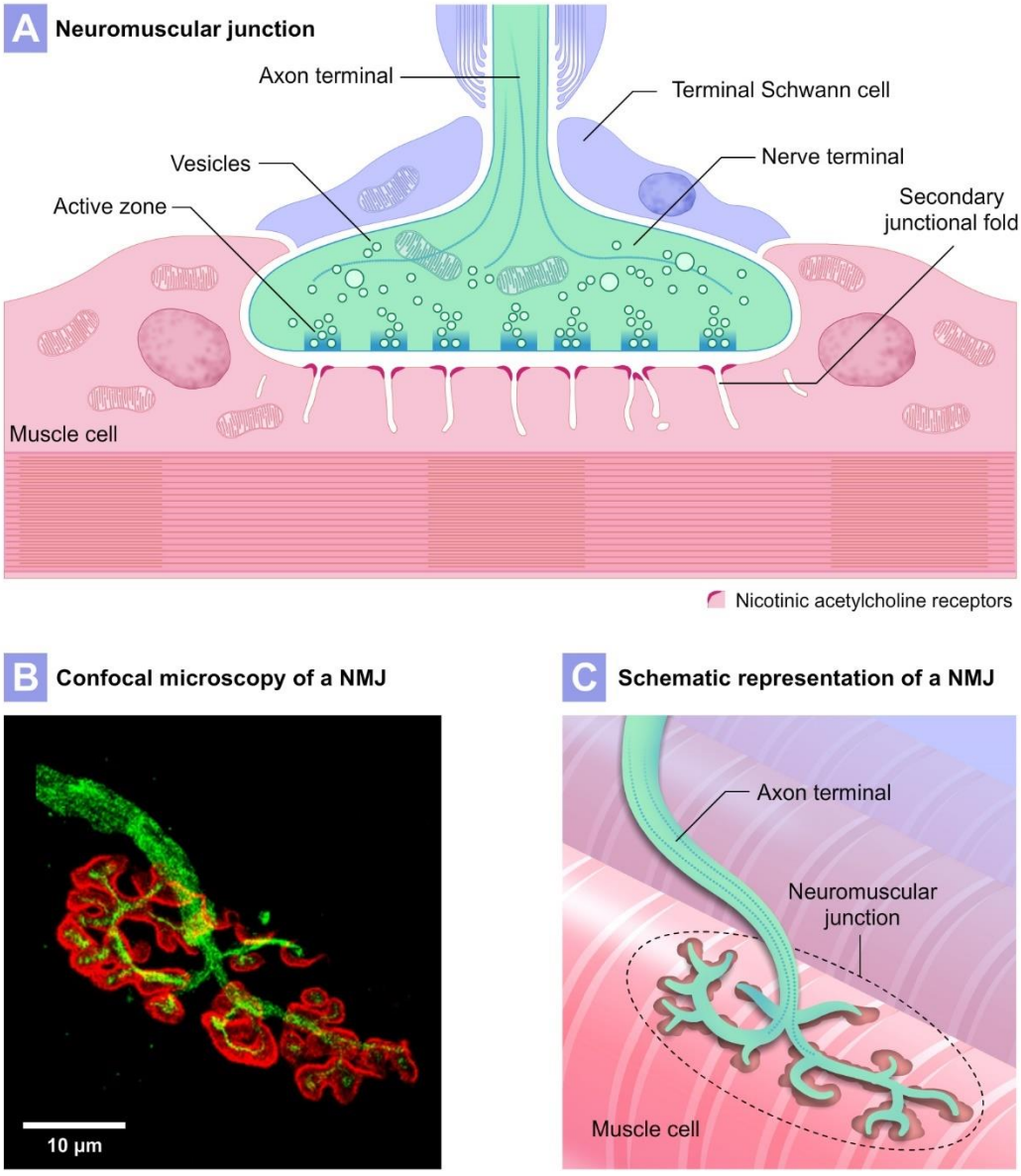

Figure 1. **Structure of the neuromuscular junction.** A The nerve terminal (in green) contains many neurotransmitter vesicles, organelles and cytoskeletal molecules. Synaptic vesicles cluster at the presynaptic membrane, forming active zones where exocytosis occurs. The perisynaptic component is constituted by the terminal Schwann cells (in blue) which, unlike axonal Schwann cells, they are not myelinogenic. Their function is to close the synaptic cleft, the space between the nerve terminal and the junctional folds of the muscle fiber. Finally, the opening area of each fold expresses nicotinic acetylcholine receptors (dark red). B Confocal microscopy of a mouse NMJ stained by immunohistochemistry. The nerve terminal has been labelled with an anti-neurofilament antibody (green) and the postsynaptic nicotinic acetylcholine receptors with α-bungarotoxin (red). C Schematic representation of the microscopy for clarity. *Source: Confocal image from Lanuza et al., (2010); Illustrations of own elaboration.*

The cytoskeleton organizes the location of the vast number of synaptic vesicles of the nerve terminal. These vesicles are concentrated near the terminal membrane in the active zones, which are specialized regions where acetylcholine release takes place. Finally, the membrane of nerve terminals is rich in ion channels and metabotropic receptors. On the one hand, sodium, potassium and calcium channels receive the signal of action potentials and trigger the neurotransmitter release. On the other, metabotropic ligand receptors present in the terminal integrate the external information and adapt the neuromuscular activity to the ongoing environment.

The muscle fiber

Muscles are contractile organs that produce motor strength to carry out movement. There are three different muscle types: smooth, cardiac and skeletal. The first two are both involuntary and constitute the walls of viscera and blood vessels (smooth), and the heart (cardiac). Skeletal muscles are part of the locomotor system, attaching to bones through tendons and allowing voluntary movement and position maintenance. The current thesis focuses on the skeletal muscle.

Skeletal muscles are formed by fascicles of long contractile tubular cells called **myocytes** or muscle fibers. These are originated from the fusion of their progenitor myoblasts, hence their multiple nuclei and extended shape.

The muscle's plasma membrane is called sarcolemma. The small area confronting the presynaptic nerve terminal at the endplate is specialized to respond to ACh. It contains the junctional folds, which express **nicotinic acetylcholine receptors** (nAChRs) at the area immediately surrounding the opening of each fold, and voltage-gated sodium channels (VGSC) at the bottom of the invagination among other molecules (■ Figure 1-A). On the other hand, the extrasynaptic membrane surrounds the rest of the muscle fiber. It contains many ion channels and digital-like invaginations, the T-tubules, that spread the stimulus from the neuron to the sarcoplasmic reticulum and the contractile fibers inside the myocyte.

Myofilaments are composed of myosin (thick) and actin (thin) protein filaments, which are responsible of muscle contraction. Grouped in bundles, myofilaments constitute myofibrils separated from each other by mitochondria and the sarcoplasmic reticulum. Thick and thin filaments alternate and give to the skeletal myocytes a striated appearance. Contraction of the muscle occurs when as thick and thin filaments slide past each other and cause the shrinkage of the muscle fiber length.

The Schwann cell

All the axons from the peripheral nervous system, myelinated and non-myelinated, are wrapped by highly specialized cells called Schwann cells (■ Figure 1). In general, axons with a low diameter are wrapped around only by the cytoplasm and one layer of plasmatic membrane of Schwann cells, designating them as amyelinic nervous fibers. Conversely, axons with a large diameter are wrapped around by a variable number of concentric layers of plasmatic membrane of Schwann cells, which generate a myelin sheath. This feature is the reason why these fibers are described as myelinated. The nerve terminals of NMJs are covered by Schwann cells that do not produce myelin and contact the myocyte to enclose the synaptic cleft. However, the immediately above Schwann cell wraps the axon in the typical myelinogenic manner. In the CNS, myelination occurs in a similar way, despite the fact that the cells which form the myelin sheaths are called oligodendrocytes.

Myelination of an axon is not continuous along its length, but occurs in small units formed by an individual Schwann cell. The myelin segment produced by each of these support cells is known as the internode. The small space between each unit of myelin is the **node of Ranvier** and has an important physiologic role in increasing the efficiency of nerve conduction. The axon at the node of Ranvier is slightly thicker than in the intermodal regions and contains most of the Na^+ gated channels of the axonal cell membrane. These gated channels are anchored via the link protein ankyrin to the cytoskeleton. On the contrary, there are no gated channels in the intermodal region beneath the myelin sheath. Whereas in the CNS the nodes

of Ranvier are uncovered, those in the peripheral nervous system are partly protected by tongue-like projections from adjacent Schwann cells.

One common characteristic of nervous cells is that conduction travels proportionately faster to the extension of the axon diameter. In this regard, myelination greatly increases the speed of conduction compared to non-myelinated fibers of the same diameter.

Schwann cells provide both structural and metabolic support to axons and NMJs. Since the soma of motoneurons is extremely distant from the processes, their homeostasis represents a great challenge. The signaling pathways that occur in Schwann cells contribute to the maintenance of healthy motoneuron activity and participate in the proliferation, differentiation and reconnection of Schwann cells during disease (Kang *et al.*, 2019; Hastings *et al.*, 2020). The long-term transport from the soma does not explain the rapid plasticity of motoneurons in front of external factors. Instead, there are pre- and postsynaptic mechanisms that tune the neuromuscular function to the ongoing situation. These are enhanced by glial cells, which provide the axon with nutrients, proteins and an mRNA pool, as well as uptake and degrade their waste products (Giuditta *et al.*, 2008; Crispino *et al.*, 2014). Finding of proteins inside the synaptic bouton of motoneurons should be considered as the result of anterograde transport and the neuron-glia interaction (Giuditta *et al.*, 2008). It is known that terminal axons can receive and gather a pool of mRNA from the surrounding glial cells (Giuditta *et al.*, 2008), accelerating their adaptation to changes without depending on the slow response from the soma. In this thesis, some findings about the levels of presynaptic proteins can be due to changes in the translation of the presynaptic terminal mRNA pool.

1.2. Function of the neuromuscular junction

Neurotransmitter release and muscle contraction

Acetylcholine is synthesized in the cytoplasm of the presynaptic nerve terminal and rapidly incorporated into small synaptic vesicles. The terminals of a single endplate can gather up to 300,000 synaptic vesicles (Guyton and Hall, 2011). When a nerve impulse reaches the neuromuscular junction, voltage-dependent calcium channels open and calcium ions (Ca^{2+}) flow into the presynaptic terminal and trigger a series of protein interactions which culminates in the fusion of the membranes of synaptic vesicles and the presynaptic terminals (Südhof, 1995; Leenders and Sheng, 2005).

Each nerve impulse causes the release 50–100 vesicles of acetylcholine from the synaptic terminal (Van der Kloot and Molgó, 1994). This occurs at specialized regions called active zones in the presynaptic terminal membrane. ACh is released into the synaptic cleft and rapidly binds to the nAChRs. These are receptors located at the tip of the postsynaptic folds, in close apposition to the presynaptic active zones. Upon ligand binding, nAChRs open their ion channel, which excites the muscle fiber membrane and ultimately causes the contraction.

The presynaptic terminals of NMJs release a greater amount of neurotransmitter–in mammals three to five times more–than that required to depolarize the postsynaptic membrane (Wood and Slater, 2001). This is called **safety factor** and ensures that muscle contraction remains reliable under various physiological conditions. The surplus of ACh is recycled by endocytosis to the nerve terminal (Heuser and Reese, 1973; Südhof, 1995). The new endosomes are transformed into synaptic vesicles, creating a **synaptic vesicle cycle** that recovers ACh and protects from depletion during stimulation. Finally, the action of ACh is terminated by **acetylcholinesterase** (AChE), an enzyme that is present in large quantities in the synaptic cleft which breaks acetylcholine a few milliseconds after it has been released from the synaptic vesicles.

Signaling pathways that modulate the neurotransmitter release

Presynaptic exocytosis is characterized by a rapid response, a short delay between excitation and secretion (Zhang *et al.*, 2002) and the limited release, with only a small percentage of docked vesicles completing fusion upon Ca^{2+} influx (Barrett and Stevens, 1972; Robitaille *et al.*, 1990; Zucker and Regehr, 2002; Ruiz *et al.*, 2011). These properties suggest that the activity of the release machinery and the docking and fusion of synaptic vesicles are tightly and finely regulated. These regulatory mechanisms require the existence of signals that, together with specific presynaptic receptors, induce intracellular signaling pathways in the nerve terminal capable of orchestrating the functional fusion machinery (Leenders and Sheng, 2005). Of special interest to our laboratory is the regulation of the neurotransmitter release by ACh and muscarinic receptors, the second messenger-activated protein kinases PKA and PKC, adenosine triphosphate (ATP) and neurotrophins, which all have been demonstrated as key regulators of the neurotransmitter release.

Acetylcholine *per se* autoregulates its release by binding to the **muscarinic acetylcholine receptors** (mAChR) (Kilbinger, 1984; Starke *et al.*, 1989). The neuromuscular junction expresses different subtypes of these metabotropic receptors, which can potentiate or inhibit the neurotransmitter release (see below).

Metabotropic receptors use downstream kinases to regulate the synaptic function. For example, the nerve terminal expresses the **protein kinase A** (PKA) and the **protein kinase C** (PKC), second messenger-activated kinases capable of regulating the release machinery through phosphorylation.

The set of receptors, kinases and other molecules involved in the synaptic release needs to be constantly maintained and refilled. Thus, the nerve ending contains many organelles that regulate protein synthesis and provide the energy source necessary for the neurotransmitter release. For example, presynaptic ribosomes and mRNA pools allow a rapid cellular adaptation to any change. This local translation at the presynaptic terminal restores the protein levels of some kinases, like PKC, when the synaptic

activity accelerates their turnover and degradation (Lee *et al.*, 1996; Lu *et al.*, 1998; Kang *et al.*, 2000; Hurtado *et al.*, 2017a).

On the other hand, mitochondria supply ATP, which is required for the synthesis of ACh, the phosphorylation activity and the release of vesicles among other cellular processes (Guyton and Hall, 2011). ATP is secreted at the NMJ and converted to adenosine in the synaptic cleft, where both molecules are sensed by the presynaptic **purinergic receptors** (Ginsborg and Hirst, 1971; Ribeiro and Sebastião, 2010). In particular, the neuromodulatory role of adenosine inhibits ACh release and preserves resources by avoiding the leak of spontaneous quantal vesicles, protecting against synaptic depression after repetitive activity at the NMJ (Ribeiro *et al.*, 2003; Tomàs *et al.*, 2014, 2018).

Finally, the postsynaptic myocyte supports the neuromuscular function by sending retrograde signaling molecules in an activity-dependent manner. Neurotrophins like the brain-derived neurotrophic factor (BDNF) are enhanced by muscle contraction (Matthews *et al.*, 2009; Hurtado *et al.*, 2017a) and received by the presynaptic terminal with the tropomyosin related kinase B (TrkB) receptor which modulates the presynaptic kinases, the proteins of the release machinery and cooperates with other receptors like mAChRs (Hurtado *et al.*, 2017a; Tomàs *et al.*, 2017; Simó *et al.*, 2018, 2019).

The study of the NMJ function

This thesis builds upon the previous knowledge on muscarinic transmission at the NMJ and attempts to provide molecular cues to the rich literature of NMJ physiology. Therefore, we considered important to briefly explain how neuromuscular transmission is recorded to understand what we know about the molecules of our interest.

The data of NMJ transmission is recorded under blocked contraction, a requirement to record the depolarization of the myocyte membrane. When a stimulus reaches the NMJ, many vesicles are released at the same time. Then, ACh molecules bind to nAChRs and cause the depolarization of the postsynaptic membrane, which is recorded as an **evoked endplate potential** (EPP). Thus, when a molecule (e.g. a muscarinic receptor or a kinase) increases (or decreases) the amplitude of the EPP, it generally means that they

modify the release machinery in a way that increases (or decreases) the number of vesicles recruited and released after the same stimulus (Fatt and Katz, 1952).

Occasionally, a single vesicle fuses spontaneously and releases a packet of transmitter (called a "quanta") in absence of any stimulation (Fatt and Katz, 1952). The small postsynaptic response to that single-vesicle exocytosis is measured as **miniature endplate potentials** (MEPPs). These events are modulated postsynaptically by changing the response to transmitter release, like changing the number of postsynaptic receptors or changing the number of acetylcholine molecules inside the vesicle. On the other hand, MEPP can also change the frequency in which they occur or the number of quanta per EPP (the quantal content), indicating some regulation of the release machinery. These parameters have revealed detailed knowledge about how receptors, kinases and other molecules participate to neurotransmission.

However, the molecular signaling pathways involved still need to be specified. In particular, the signaling related to the modulation of the ACh release by the muscarinic receptors through protein kinases A and C at the NMJ. In the following sections of this introduction, we show the state of art of main molecules of this signaling pathway.

2. Muscarinic signaling at the NMJ

2.1. The muscarinic receptors

Muscarinic classification and pathways

Acetylcholine release at the NMJ activates both the ionotropic **nicotinic receptor** (nAChR) and the metabotropic **muscarinic receptor** (mAChR). nAChRs are ligand-gated ion channels whereas mAChRs belong to the superfamily of G protein-coupled receptors (GPCRs) and modulate the action of a particular G-protein (■ Figure 2-A).

Mammals express five subtypes of mAChRs, termed M_1–M_5 (Caulfield and Birdsall, 1998), and encoded in the genes CHRM1 to CHRM5 (Fredriksson *et al.*, 2003). These five mAChR subtypes can be classified into two groups according to their signaling: odd-numbered mAChRs (M_1, M_3 and M_5) preferentially couple $G_{q/11}$-type G-proteins; and even-numbered mAChRs (M_2 and M_4) activate $G_{i/o}$-type G-proteins (■ Figure 2-B). Because the $G_{i/o}$ protein is affected by the pertussis toxin (PTX), sometimes the M_1, M_3 and M_5 subtypes are referred as PTX-insensitive, whereas M_2 and M_4 as PTX-sensitive (Minic *et al.*, 2002). A common tool to classify and study the properties of muscarinic receptors subtypes are muscarinic antagonists. Among them stand out **pirenzepine** (Pir) for M_1 blockade; **methoctramine** (Met) for M_2 blockade; 1,1-dimethyl-4-diphenylacetoxipiperidinium (4-DAMP) for M_3 blockade; MT-3 and tropicamide for M_4 blockade and **atropine** (Atr) for general muscarinic blockade (Caulfield and Birdsall, 1998).

Muscarinic receptors induce numerous signaling pathways that confluence to the second-messenger kinases PKC and PKA (■ Figure 2-C). M_1, M_3 and M_5 receptors activate the **phospholipase C beta** (PLCβ) through the alpha subunit of $G_{q/11}$ protein, mainly PLCβ1 and β4 subtypes (Ross and Berstein, 1993; Biddlecome *et al.*, 1996; Nathanson, 2000; Strassheim and Williams, 2000). It should be noted that muscarinic signaling is highly complex and that other PLCβ isoforms, namely PLCβ2 and β3, can be recruited by M_2 and M_4 subtypes through the $G_{\beta\gamma}$ subunit (Nathanson, 2000). In turn, PLC selectively hydrolyzes the membrane phospholipid phosphatidylinositol-4,5-

bisphosphate (PIP_2) and produces diacylglycerol (DAG) and inositol trisphosphate (IP_3). The latter path induces the mobilization of intracellular calcium ions (Ca^{2+}) and activates PKC and the phosphorylation of its targets. On the other hand, M_2 and M_4 receptors inhibit adenylyl cyclase (AC) through the $G_{i/o}$-type protein (Caulfield and Birdsall, 1998). This decreases the intracellular concentration of 3',5'-cyclic adenosine monophosphate (cAMP) and inhibits PKA. Finally, the Gβγ subunit of mAChRs can also induce its own signaling. For example, in portal vein myocytes, M_2 mAChR uses the Gβγ subunit to activate the phosphoinositide 3-kinase (PI3K) (Wang *et al.*, 1999; Callaghan *et al.*, 2004). PI3K phosphorylates the membrane phospholipid PIP_2 and produces phosphatidylinositol-3,4,5-bisphosphate (PIP_3). In turn, this molecule enhances the activity of 3-phosphoinositide dependent kinase 1 (PDK1) which is a master kinase controlling several others including PKC (Toker, 2003; Mora *et al.*, 2004; Bayascas, 2010; Pearce *et al.*, 2010).

In this thesis, we investigated the muscarinic signaling at the NMJ, focusing on the role of PKC and PKA kinases and how they interact with their respective regulatory proteins and substrates of the exocytotic machinery.

Receptor activation and regulation

In the periphery, the activity of muscarinic receptors mediates smooth muscle contraction, glandular secretion, and modulation of cardiac rate and force. In the CNS, muscarinic receptors are involved in motor control, temperature regulation, cardiovascular regulation and memory.

The first mechanism that controls the activation of muscarinic receptor subtypes is their **affinity for their ligand, acetylcholine.** Muscarinic affinity is studied using orthosteric muscarinic compounds like tritiated quinuclidinyl benzylate [^{3}H]QNB or N-methylscopolamine [^{3}H]NMS (Lazareno *et al.*, 2004; Haga, 2013). For example, on membranes from CHO cells that express a particular muscarinic receptor subtype, [^{3}H]NMS has higher affinity for M_2 and M_2-NMS complexes are highly inhibited by acetylcholine (Lazareno *et al.*, 2004). At the neuromuscular junction, the sensitivity to ACh has been classically measured with functional experiments. Minic *et al.* (2002) determined that the

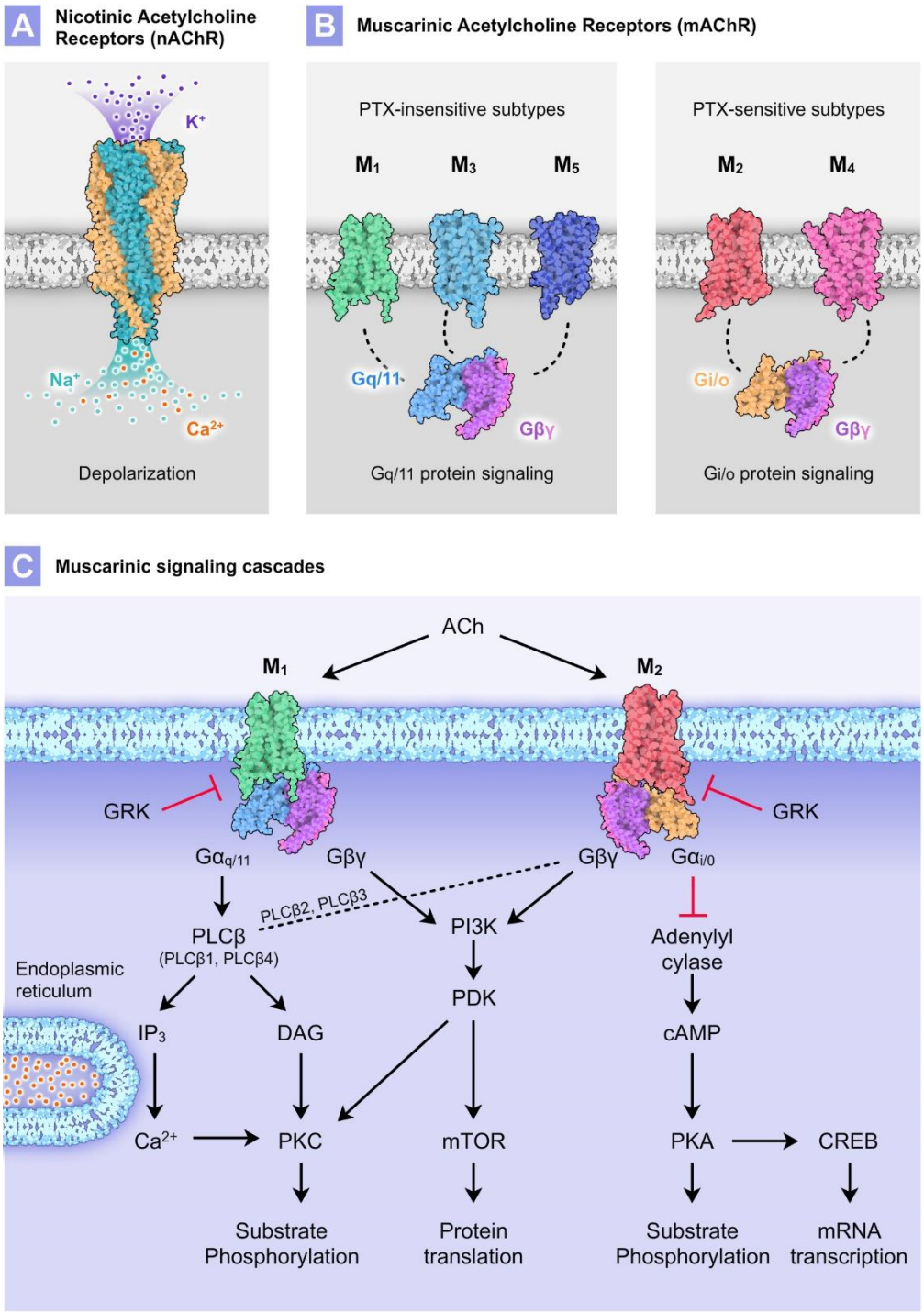

Figure 2. **Acetylcholine receptors and muscarinic signaling.** A Nicotinic acetylcholine receptors (nAChRs) induce their intracellular signaling through membrane depolarization. B Muscarinic receptors (mAChRs) are a family of five subtypes: M_1, M_3 and M_5 couple to $G_{q/11}$-type G proteins, whereas M_2 and M_4 couple $G_{i/o}$-type G proteins. C Muscarinic receptors induce numerous signaling pathways that confluence to the second-messenger kinases PKC and PKA. The details of the signaling are explained in the text. *Source: own elaboration.*

M_1-M_2 balance at the NMJ is sensitive to the ACh levels by modulating acetylcholine esterase. They showed that at normal/low neurotransmission levels at the NMJ, muscarinic receptors inhibit neurotransmission because muscarine decreases the quantal content (Minic *et al.*, 2002). Using specific blockers, they could determine that M_2 was responsible for this action. Additionally, increasing ACh concentration in the synaptic cleft unmasked the effect of M_1 muscarinic receptor, which increased the quantal content, indicating a lower affinity for ACh (Minic *et al.*, 2002). Similar approaches are concordant: the overall muscarinic signaling inhibits neurotransmission (M_2 overcomes M_1) because the general inhibitor atropine increases the quantal content (Slutsky *et al.*, 1999; Santafé *et al.*, 2006). Thus, radiolabeling experiments and *in vivo* findings indicate that M_2 has more affinity for ACh than M_1 and that M_2 inhibitory signaling predominates under standard conditions and low impulse frequencies.

Muscarinic receptors are also regulated by **phosphorylation**, which leads to desensitization in some cases. The phosphorylation occurs on Ser/Thr residues of their cytoplasmic portion, corresponding to the third cytoplasmic loop and C-terminus (van Koppen and Kaiser, 2003). The kinases able to phosphorylate mAChRs are GPCR kinases (**GRKs**), casein kinase 1α and PKC (van Koppen and Kaiser, 2003; Haga, 2013). GRKs are the main modulators of muscarinic internalization. They only phosphorylate agonist-occupied mAChRs (Haga *et al.*, 1996), leading to the **β-arrestin/dynamin dependent internalization** and termination of the signal (■ Figure 2-C)(Krasel *et al.*, 2001; Penela *et al.*, 2003). At basal conditions, GRKs are cytosolic kinases that are recruited to the plasma membrane by binding to the Gβγ subunit and PIP_2 in response to agonist activation (van Koppen and Kaiser, 2003; Haga, 2013). On the other hand, the casein kinase 1α phosphorylates M_3 mAChRs receptors and seems to promote the M_3-mediated activation of the mitogen-activated protein kinase pathway (Budd *et al.*, 2001). Of particular interest for this thesis are PKA and PKC. Most studies have failed to show the involvement of PKA and mAChR internalization. Treatments with PKA inhibitors, activators or forskolin (adenylyl cyclase stimulator) do not induce mAChR internalization (Koppen *et al.*, 1995). In regard to PKC, it is known to

phosphorylate M_1 and M_3 but not M_2 mAChRs (Haga et al., 1990; van Koppen and Kaiser, 2003). PKC likely phosphorylates the residues Thr^{354} and Ser^{356} of the third cytoplasmic loop and Ser^{451}, Thr^{455} and Ser^{457} of the C-terminus of M_1 mAChR (Haga *et al.*, 1996). However, these PKC phosphorylations do not demonstrate activation or induce receptor internalization. This is because PKC-mediated mAChR phosphorylation is independent of agonist, independent of Gβγ and does not affect surface or total muscarinic receptor number in most cell systems (van Koppen and Kaiser, 2003). Instead, phosphorylated mAChRs seem more linked to their inhibition, as phorbol esters block mAChR-mediated calcium increase, PIP_2 turnover and inhibition of cAMP accumulation in several nervous cell cultures (Kanba *et al.*, 1986; Lai *et al.*, 1990; van Koppen and Kaiser, 2003).

Finally, muscarinic receptors can be regulated in the long term by modulating their **synthesis**. mAChRs undergo down-regulation in response to agonist-induced sustained activation (Hao *et al.*, 2005). Therefore, therapeutic effectiveness decreases during continuous use. In cell cultures, M_1 receptor number is upregulated through a cAMP pathway-mediated stimulation of gene transcription (Hao *et al.*, 2005). On the other hand, methoctramine and other muscarinic antagonists upregulate the levels of M_2 and other subtypes of mAChRs (Ben-Barak and Dudai, 1980; Haddad and Rousell, 1998).

2.2. Muscarinic receptors at the neuromuscular junction

Muscarinic expression at the NMJ

The three cellular components of the neuromuscular junction work hand in hand to achieve a proper synaptic function. Each of these cells has a specialized set of proteins and mediators. This unique environment explains how one protein can play different roles depending on the cellular context where it is found. Thus, the expression and distribution of muscarinic receptors is important to understand their function. In our laboratory, Garcia *et al.*, 2005 did the first immunohistochemical localization of muscarinic receptors at the NMJs of *Levator auris longus* (LAL) muscles from newborn

and adult rats. In adult muscles, M_1, M_2, M_3 and M_4 mAChR subtypes were expressed in the vicinity of NMJs (M_5 was not investigated) (■ Figure 3).

The first evidence of M_1 location at the NMJ was the use of fluorescent-conjugated MT-7 (M_1 blocker), suggesting the presence of presynaptic M_1 mAChRs in the synaptic area, without distinguishing whether the receptors were localized in motor nerve terminals and/or perisynaptic Schwann cells (Minic *et al.*, 2002). Using immunohistochemistry, M_1 receptors were mainly found in the axon and the nerve terminal of adult rat NMJs, colocalizing with syntaxin (Garcia *et al.*, 2005). Furthermore, M_1 immunoreactivity appeared outside the borders of nAChRs and syntaxin, suggesting the presence of the receptor in the teloglial Schwann cells (Garcia *et al.*, 2005). A similar M_1 distribution was demonstrated at the rat diaphragm through electron microscopy by Malomouzh *et al.*, 2011. M_1 muscarinic receptors were found at the presynaptic membrane of the nerve terminal and at the postsynaptic membrane of skeletal muscle, mostly in the depths of postsynaptic folds (Malomouzh *et al.*, 2011). However, in that study M_1 immunoreactivity was not detected perisynaptically or outside the synaptic contact. These observational differences of M_1 immunoreactivity at teloglial cells and the postsynaptic muscle may be due to the different primary antibodies used, the type of muscle and the low density of muscarinic receptors (Malomouzh *et al.*, 2011). Overall, M_1 receptors can be found in all three components of the NMJ.

Regarding M_2 mAChRs, its expression is concentrated around the NMJ endplates from adult LAL muscles (Garcia *et al.*, 2005). Unlike M_1, anti-M_2 labelling (Alomone) does not stain the preterminal axon. Instead, M_2 area extends beyond the borders of syntaxin and nAChRs, suggesting teloglial cell labeling. In particular, oval areas of low mAChR intensity were observed, most likely occupied by Schwann cells nuclei (Garcia *et al.*, 2005). A subsequent immunohistochemistry study found that M_2 mAChRs are expressed selectively in motoneurons (Wright et al., 2009). In particular, their research found that an anti-M_2 antibody (also Alomone, distinct from the one used in the present thesis) labels the three NMJ cell components and, in $M_2^{-/-}$ knockout mice, only the nerve terminal labelling disappears.

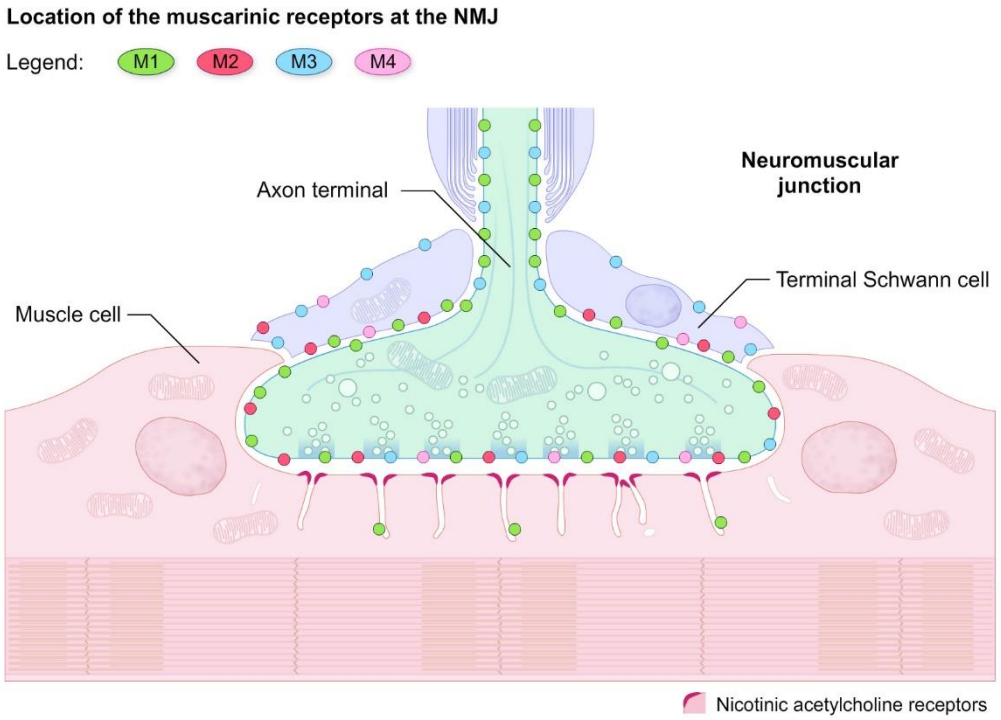

Figure 3. **Location of muscarinic receptors at the NMJ.** In the adult neuromuscular junction, all muscarinic receptors are enriched near the synaptic area. The M_1 receptors (green) have been localized in the presynaptic terminal with three methods (fluorescent-conjugated MT-7, fluorescent-conjugated antibodies and electron microscopy) (Minic *et al.* 2002; Garcia *et al.*, 2005; Malomouzh *et al.*, 2011). Besides the nerve terminal labelling, the first two papers observed perisynaptic labelling–Schwann cells–and the third observed M_1 labelling in the depths of postsynaptic folds. The M_2 receptor (red) is concentrated around NMJ endplates but it is not present in the axon branch. Two studies confirm presynaptic expression with fluorescent immunolabelling (Garcia *et al.*, 2005; Wright *et al.*, 2009). Perisynaptic location is unlikely as genetic studies reject M_2 labelling from Schwann cells. The M_3 receptors (sky blue) have been found in the synaptic area and axons whereas M_4 receptors (pink) are only present near the presynapse (Garcia *et al.*, 2005). *Source: own elaboration.*

Muscarinic signaling at the NMJ

In the last decades, our laboratory studied in detail the function of mAChRs at the NMJ. Table 1 summarizes the main electrophysiological findings of muscarinic modulators alone. The M_1 and M_2 mAChRs are the major muscarinic subtypes involved in the enhancement and inhibition, respectively, of ACh release at the adult NMJ (frog: Slutsky *et al.*, 1999, mouse: Minic *et al.*, 2002 and rat: Santafé *et al.*, 2003). In these studies, M_3 and M_4-selective antagonists did not modify ACh release. Instead, M_3 and M_4 subtypes only modulated neurotransmission during the developmental stage of newborn NMJs (Santafé *et al.*, 2004; Nadal *et al.*, 2016, 2017).

Double-inhibitor experiments showed that the inhibition of neurotransmitter release by pirenzepine (M_1 inhibitor) can be recovered if methoctramine (M_2 inhibitor) is added to the media, raising back the amplitude of the release (Santafé *et al.*, 2007). The same happens vice versa, which demonstrates that M_1 and M_2 pathways use, in general, different independent mechanisms (Santafé *et al.*, 2007). Therefore, the presynaptic co-expression of both mAChRs and their ability to cancel each other out creates a regulatory platform that can be balanced through multiple pathways (Minic *et al.*, 2002; Santafé *et al.*, 2003).

In multiple synapse models, the function specificity of mAChRs was classically simplified as the M_1, M_3 and M_5 receptors activating PLCβ/PKC whereas M_2 and M_4 receptors inhibiting AC/PKA (Caulfield and Birdsall, 1998). However, the current vision knows that muscarinic signaling is complex and interconnected at several kinases (Nathanson, 2000). At the NMJ, our laboratory demonstrated by electrophysiology techniques that both M_1 and M_2 mAChRs involve PKC to modulate transmitter release (Santafé *et al.*, 2006). In particular, M_1, M_3 and M_5 action on PKC is directly mediated by PLC activation; and M_2 and M_4 action is indirectly mediated by inhibition of AC/PKA, which also results in PKC inhibition (Santafé *et al.*, 2009). Other experiments at the NMJ also find connections between both mAChR subtypes. For example, when the media contains a high concentration of magnesium ions (Mg^{2+} 5 mM) or exogenous AChE, M_2 shifts to potentiate ACh release like M_1 (Santafé *et al.*, 2007). The high Mg^{2+} concentration interferes with calcium/PKC signaling, and thus may uncover an underneath M_2 pathway.

Besides kinases, voltage-gated calcium channels (VGCC) also participate in the muscarinic modulation of transmitter release (Santafé *et al.*, 2003, 2004). At the adult NMJ, M_1 mAChR enhances acetylcholine release through a P/Q-type VGCC-dependent mechanism, whereas M_2 mAChR inhibits the release and depends on the availability of sufficient internal calcium, rather than involving VGCCs (Santafé *et al.*, 2003).

Table 1. Muscarinic electrophysiology at the adult NMJ. Current knowledge from electrophysiology experiments in frog (Slutsky *et al.*, 1999) and rat muscle preparations (Santafé *et al.*, 2003; 2004; 2007) testing muscarinic modulators. Abbreviations: EPP, evoked endplate potential.

Pretreatment (30 minutes)	Muscarinic modulator (subtype target)	Effect on EPP amplitude	Reference
None (physiological condition)	Pirenzepine (M_1)	Decrease. M_1 enhances the release.	Slutsky (1999) Santafé (2003)
	MT-7 (M_1)		Santafé (2004)
	Methoctramine (M_2)	Increase. M_2 inhibits the release.	Slutsky (1999) Santafé (2003)
	AF-DX 116 (M_2)		Santafé (2004)
	4-DAMP (M_3)	No effect. M_3 does not modulate the release at the adult NMJ.	Santafé (2003)
	Tropicamide (M_4)	No effect. M_4 does not modulate the release at the adult NMJ.	Santafé (2003)
	MT-3 (M_4)		Santafé (2004)
	Muscarine (M_1-M_5)	Decrease. Overall muscarinic potentiation inhibits the release (i.e. $M_2 > M_1$).	Slutsky (1999)
	Atropine (M_1-M_5)	Increase. Overall muscarinic inhibition enhances the release (i.e. $M_2 > M_1$).	Santafé (2003)
Pirenzepine + Methoctramine	Muscarine	No effect. M_1 and M_2 receptors are the major subtypes modulating the release.	Slutsky (1999)
Pirenzepine	Methoctramine	Decrease. M_2 uses a pathway independent of M_1.	Santafé (2007)
Methoctramine	Pirenzepine	Increase. M_1 uses a pathway independent of M_2.	Santafé (2007)
High Mg^{2+}	–	Decrease. Magnesium competes with calcium and inhibits the release.	Santafé (2007)
	Pirenzepine	Decrease. In low release conditions, M_1 still potentiates the release.	Santafé (2007)
	Methoctramine	Decrease. In low release conditions, , affects the M_2 pathway.	Santafé (2007)
	Atropine	Decrease. High magnesium media affects the overall muscarinic signaling.	Santafé (2007)
Exogenous AChE	–	Decrease. AChE degrades ACh and inhibits the release.	Santafé (2007)
	Pirenzepine	Decrease. In low release conditions, M_1 still potentiates the release.	Santafé (2007)
	Methoctramine	Decrease. Low ACh shifts M_2 to potentiate the release.	Santafé (2007)
	Atropine	Decrease	Santafé (2007)

3. Protein kinase A and cAMP signaling

The 3',5'-cyclic adenosine monophosphate (cAMP) modulates numerous cellular processes such as cell growth and differentiation, the synaptic release of neurotransmitters, ion channel conductivity and gene transcription. The principal intracellular target for cAMP in mammalian cells is the cAMP-dependent protein kinase (PKA).

3.1. The protein kinase A

The cAMP-dependent protein kinase is a serine/threonine kinase also known as protein kinase A, cAPK or PKA. It is one of the most important kinases and performs a central role in the signaling pathway of many cell types (Hancock, 2016). In neuromuscular cells, its actions include the activation of neurotransmission in motoneurons, the regulation of metabolic rate in the skeletal myocyte and, more in general, the control of gene expression.

PKA is a tetramer of two catalytic and two regulatory subunits

As its name suggests, cAMP-dependent protein kinase is controlled by the levels of cAMP in the cell. When cAMP levels are low, PKA stays in its inactive state, a protein tetramer of two **catalytic subunits** (C) associated with two **regulatory subunits** (R)(■ Figure 4-A)(Hancock, 2016). When the cAMP levels rise, two cAMP molecules bind to each R subunit and cause a conformational change which dissociates the complex. Then, the C subunits are liberated and start their catalytic activity whereas the R subunits remain as a dimer (Walsh *et al.*, 1968; Beavo *et al.*, 1975; Taylor *et al.*, 2012).

Murine models express four R subunit isoforms (RIα, RIβ, RIIα, RIIβ) and two C subunit isoforms (Cα, Cβ); whereas the Cγ gene is only found in primates (■ Figure 4-A)(Beebe *et al.*, 1990; Reinton *et al.*, 1998). R subunits **homodimerize** through interactions at their N-terminus, generating the holoenzymes $RI\alpha_2C_2$, $RI\beta_2C_2$, $RII\alpha_2C_2$ or $RII\beta_2C_2$ (■ Figure 4-B). Generally, there is no preference of association between certain R and C subunits (Brandon *et al.*, 1997). Cα and Cβ subunits display essentially the same activation properties in *in vitro* purified complexes (Cadd et al., 1990).

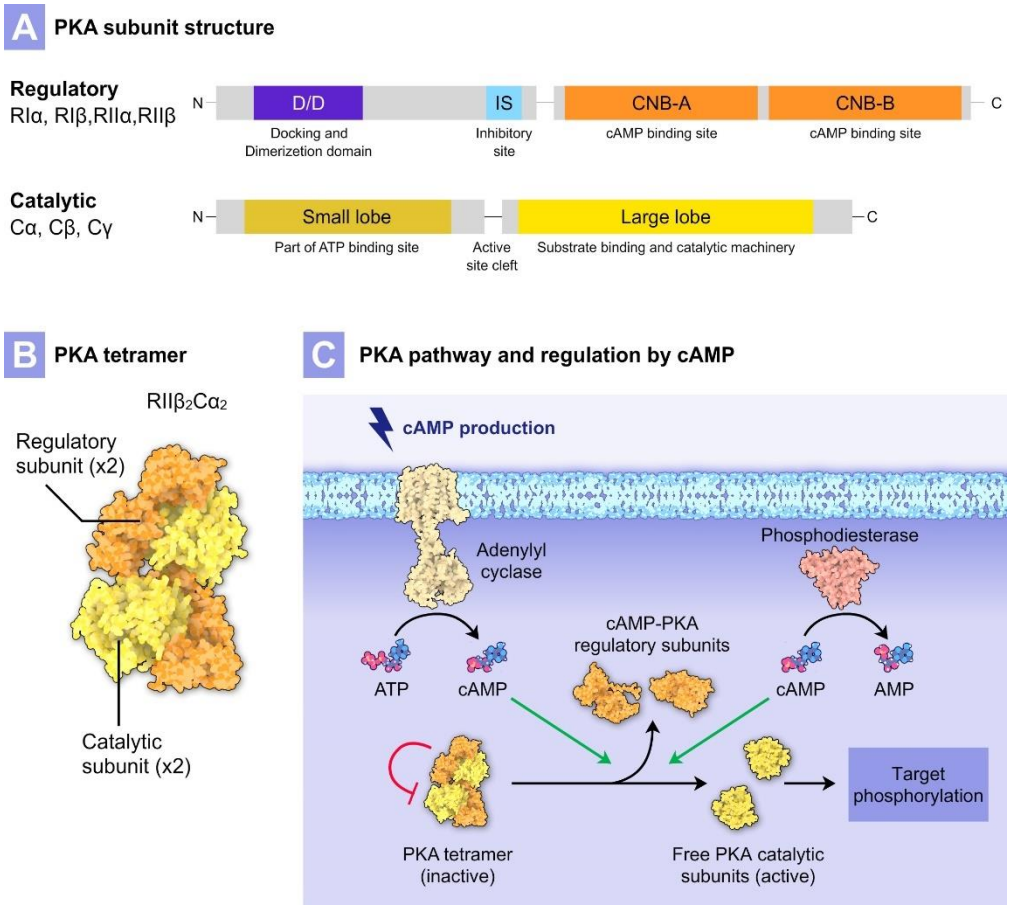

Figure 4. **The PKA kinase.** A Protein kinase A (PKA) has two classes of subunits: regulatory (RIα, RIβ, RIIα and RIIβ) and catalytic (Cα and Cβ), very similar in sequence between them. Regulatory subunits have one docking and dimerization domain (D/D) which induces their homodimerization, an inhibitory site (IS) to block PKA activity and two cyclic nucleotide binding (CNB) domains. Catalytic subunits have two main lobes surrounding their active site. B PKA subunits interact with each other to form a tetramer, where regulatory subunits inhibit the activity of catalytic subunits. C The PKA pathway is activated by adenylyl cyclase, which produces cAMP and triggers PKA complex dissociation and catalytic activity. This signal is terminated by phosphodiesterases. Abbreviations: ATP, adenosine triphosphate; cAMP, 3',5'-cyclic adenosine monophosphate. *Source: Adapted from Newton et al. (2008).*

Regarding type-I subunits, holoenzymes containing the neural RIβ are more sensitive to cAMP-evoked activation than RIα-containing holoenzymes (Cadd et al., 1990). Also, although RII holoenzymes seem more prone to dissociate than RI, probably due to structural differences, RI are more efficient than RII in inducing CREB response regardless of the C subunit (Vigil *et al.*, 2004; Prinz *et al.*, 2006; Stakkestad *et al.*, 2011).

PKA activation and regulation

After binding cAMP, PKA catalytic subunits phosphorylate the next protein to continue the cAMP signaling cascade (■ Figure 4-C). This kinase usually phosphorylates peptides that contain two consecutive basic residues (arginine or lysine) at positions 2 and 3 from the N-terminal site of a serine (Ser) or a threonine (Thr):

-Arg-Arg-X-Ser-X- -Arg-Arg-X-Thr-X-

The first residue termed "X" is usually a small amino acid, whereas the second "X" residue is usually hydrophobic (Hancock, 2016). The presence of this consensus site can be used to identify possible PKA targets. In this thesis we studied the PKA targets CREB, whose PKA sequence lies around serine 133 (Ser^{133}), and SNAP-25, around threonine 138 (Thr^{138}).

PKA phosphorylation regulates gene expression by activating transcription factors such as the **CRE-binding protein** (**CREB**) (Hancock, 2016). When PKA phosphorylates CREB at serine 133 (Ser^{133}), pCREB becomes active and binds to CRE (cAMP-response element) regions of the DNA (Montminy, 1997). Some genes containing consensus CREB-binding sites in their upstream promoter encode for neuropeptides such as the brain-derived neurotrophic factor (BDNF) (Mayr and Montminy, 2001) and the neurotransmission-related proteins such as synapsin-1 (Montminy, 1997).

The attenuation of cAMP/PKA effect is coordinated by cyclic nucleotide **phosphodiesterases** (PDEs). In skeletal muscle, PDE4 appears to contribute to the majority of cAMP hydrolysis, accounting for >80% of the total PDE activity in the tissue (Bloom, 2002). On the other hand, PKA can also be regulated by **changes in its own concentration** through synthesis and degradation (Spaulding, 1993; Garrel *et al.*, 1995) and by crosstalk with the PKC pathway at the NMJ (Santafé *et al.*, 2009) and with other systems (Cronin *et al.*, 1986; Summers and Cronin, 1986; Barnes and Conn, 1993; Garrel *et al.*, 1995), although the molecular signaling involved is unknown. Finally, PKA activity and specificity is also modulated through the subcellular location of the subunits, which is controlled by scaffold proteins (Colledge and Scott, 1999).

PKA scaffolding: role of AKAP150

Time and cellular location of enzymes are crucial to either amplify or decrease their signal transduction pathway. **Scaffold proteins** are polypeptides whose function is to anchor target proteins (usually kinases and phosphatases) to the right subcellular environment. Even though scaffold proteins may not have inherent activity, they provide a temporal and spatial regulation by concentrating enzymes near neighboring substrates (Wong and Scott, 2004; Hancock, 2016).

The A-kinase-anchoring proteins (**AKAPs**) are a family of scaffolding proteins that compartmentalize PKA and other enzymes. Subtle differences between family members direct each AKAP to distinct cell compartments (Wong and Scott, 2004). In rodents, AKAP150 (equivalent to the human homologue AKAP79) is anchored to the plasma membrane through sequences that bind phospholipids. AKAP150 participates in neuronal function, as it is both present in postsynaptic density fractions of neuronal lysates and in the dendritic spines of neurons. AKAP150 maintains PKA, PKC, and protein phosphatase 2B at the postsynaptic density of mammalian synapses (Perkins *et al.*, 2001; Zeng *et al.*, 2014).

Therefore, PKA activity is regulated by subcellular targeting besides cAMP activation (Constantinescu *et al.*, 2002; Gervasi *et al.*, 2007). Both PKA C subunits (Tillo *et al.*, 2017) and R subunits (Reinitz *et al.*, 1997; Stefan *et al.*, 2011) translocate between cytosol and membrane to regulate their activity. When inactive, most PKA tetramers are anchored to the membrane by the interaction of R subunits with AKAPs. These AKAPs contribute to PKA specificity by recruiting the PKA holoenzyme to distinct subcellular compartments near specific substrates (Lohmann *et al.*, 1984; Wong and Scott, 2004; Scott and Pawson, 2009). R subunits have different subcellular location, RI being present mainly in the cytosol, whereas RII mainly in the particulate fraction, associated with the nuclei, nucleoli, Golgi complex and microtubules (Brandon *et al.*, 1997; Keryer *et al.*, 1999).

3.2. PKA at the neuromuscular junction

PKA location at the neuromuscular junction

The PKA R subunits are differently expressed across tissues and exert distinct roles in cell differentiation and growth control (Skålhegg and Tasken, 2000; Fagerberg *et al.*, 2014). Unfortunately, the localization of specific PKA subunits at the NMJ remains elusive due to their sequence similarity and the ubiquity of PKA expression. Therefore, localization experiments depend on the species, the muscle studied and the specificity of the antibodies or the probes used. The Figure 5 summarizes the location of PKA regulatory subunits at the NMJ.

The RIα subunit is widely expressed. It is found enriched in the cytosolic fraction of different muscle groups studied (Hoover *et al.*, 2001; Perkins *et al.*, 2001). Histologically, RIα expression concentrates around NMJs. In the postsynaptic component, RIα colocalizes with nAChRs (Imaizumi-Scherrer *et al.*, 1996; Perkins *et al.*, 2001) and directly interacts with nAChRs in the *tibialis anterior* muscle (Röder *et al.*, 2010). This labelling expands further than the NMJ and, within the myocyte, RIα remains associated with actin microfilaments (I-band) and mitochondria (Perkins *et al.*, 2001; Rudolf *et al.*, 2013). However, RIα is also present at the presynaptic component, being colocalized with the presynaptic vesicle marker synaptophysin, and also observed in other vicinities of the presynapse (Perkins *et al.*, 2001). In the rat *tibialis anterior* and extraocular muscles, the mRNA of RIα was found enriched at the NMJ in comparison to the nearby NMJ-free fiber regions (Ketterer *et al.*, 2010).

The expression of the regulatory RIβ subunit is more restricted to nervous tissues such as the spinal cord and the brain (Cadd and McKnight, 1989; Skålhegg and Tasken, 2000). The first localization of the RIβ subunit at the neuromuscular junction has been done recently in our laboratory, finding RIβ close to the synapse and present in all three cells of mice NMJs during development (Garcia *et al.*, 2019).

The regulatory RIIα subunit is abundant and widely expressed, predominantly in cardiac muscle fibers (Skålhegg and Tasken, 2000). In the rat

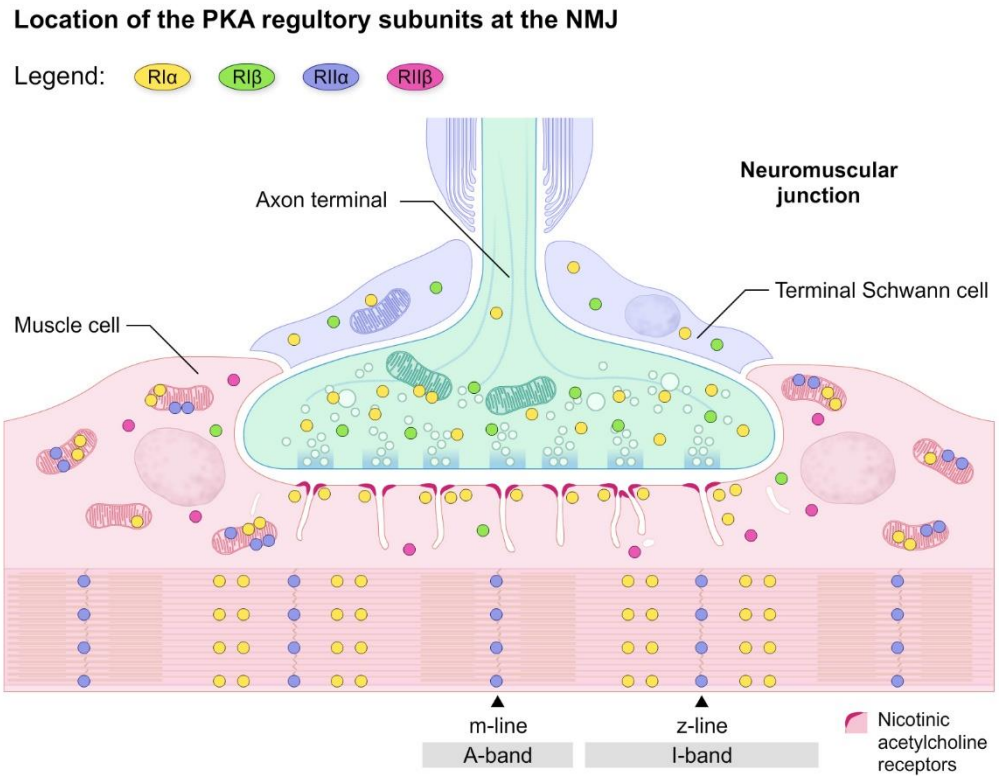

Figure 5. **Location of PKA R subunits at the NMJ.** The regulatory RIα subunit (yellow) is ubiquitously expressed in the three cell components of the NMJ. In the postsynaptic component, it associates with nicotinic acetylcholine receptors and mitochondria. The RIβ subunit (green) is generally predominant in the nervous tissue. Data from NMJ has only been obtained in development stage, where it is observed in all the three components of the NMJs. RIIα (blue) is abundant and concentrated in the postsynaptic region and colocalizes with mitochondria, m-lines and z-lines, but not with nicotinic acetylcholine receptors. Finally, RIIβ is scarce and sometimes not detected in muscle. At the NMJ, RIIβ has been located at the cytosolic fraction and associated with a broader area of the postsynaptic region but absent in the rest of the muscle fiber. *Source: own elaboration.*

intercostal muscle, RIIα concentrates around the postsynaptic regions of NMJs, colocalizing with mitochondria but not with nAChRs (Perkins *et al.*, 2001). Outside the NMJ, RIIα labels the z-lines and m-lines from muscle fibers, regions where mitochondria are present (Perkins *et al.*, 2001; Röder *et al.*, 2009; Rudolf *et al.*, 2013). Further studies are needed to clarify if the abundant RIIα is also present in the presynaptic site.

The regulatory RIIβ is probably the scarcest PKA subunit in the muscle tissue. One study reported no expression in the soleus and *extensor digitorum longus* muscles (Hoover *et al.*, 2001) whereas another found it in rat intercostal muscle (Perkins *et al.*, 2001), possibly due to the presence of adipocytes and nerve fibers where it is prevalent (Cadd and McKnight, 1989). At

the cellular level, RIIβ is present in the membrane and the cytosolic fractions, but mostly associated with the latter (Perkins *et al.*, 2001). Histologically, RIIβ immunoreactivity is observed in the postsynaptic component near the NMJ, associated with a broader area, which does not overlap with nAChRs (Perkins *et al.*, 2001). Besides the synapses, Perkins and cols. found a lack of labelling of RIIβ in the rest of the muscle fiber. This is in agreement with the scarcity and lack of RIIβ mRNA hybridization in the skeletal muscle (Imaizumi-Scherrer *et al.*, 1996). However, due to the lack of high-resolution studies, RIIβ presence in the presynaptic site cannot be ruled out.

The localization of PKA Cα and Cβ catalytic subunits at the NMJ has not been explored and is part of the objectives of this thesis. Nevertheless, data from the CNS suggests that PKA Cβ subunit is a good candidate to mediate muscarinic signaling, as it is primarily expressed in brain regions and linked to neuronal functions. (Brandon *et al.*, 1997).

PKA modulation of acetylcholine release

The PKA enhances the release at the adult NMJ at basal conditions (Santafé *et al.*, 2009). This can be concluded because the PKA inhibitor **H-89** decreases the amplitude of the evoked endplate potentials, whereas the activator Sp-8-Br-cAMP increases it (**Table 2**). Nevertheless, there seems to be more than one mechanism by which PKA increases the synaptic release (Santafé *et al.*, 2009; Gaydukov *et al.*, 2019). This has been explored by studying how the kinase affects the exocytosis of single synaptic vesicles i.e., MEPPs (See Section *The study of the NMJ function*).

The first studies at the frog NMJ found that cAMP and other PKA analogues increased the amount of ACh being released in each vesicle (Van der Kloot and Branisteanu, 1992). The outcome was different in mammalian NMJs. Our laboratory found that stimulating PKA at the rat LAL muscle with Sp-8-Br-cAMP (10 μM) did not increase the quantal size after 1 and 3 hours (Santafé *et al.*, 2009). This indicates that the action of PKA does not affect postsynaptic nAChRs or vesicle filling over the timescale studied at the rat NMJ. However, PKA stimulation increased MEPP frequency, showing that PKA modifies the presynaptic neurotransmission machinery to make it more prone to release.

Table 2. PKA action on the release of the adult NMJ. Current knowledge of from electrophysiological experiments testing PKA modulators. Abbreviations: EPP, evoked endplate potential.

Pretreatment (1)	PKA treatment (2)	Effect on EPP amplitude (2 vs 1)	Reference
Control (physiological conditions)	H-89	Decrease. PKA promotes ACh release.	Santafé (2009)
	Sp-8-Br-cAMP	Increase. PKA promotes ACh release.	Santafé (2009)
CaC (PKC blockade)	H-89	Decrease. PKA is still active after PKC inhibition.	Santafé (2009)
	Sp-8-Br-cAMP	Increase. PKA does not need PKC activity to promote ACh release.	Santafé (2009)
High Ca^{2+}	H-89	Decrease. PKA is still active after Ca^{2+}/PKC activation.	Santafé (2007)
	Sp-8-Br-cAMPs	None. PKA cannot be further stimulated after high Ca^{2+}.	Santafé (2007)
PMA (PKC activation)	H-89	Decrease. PKA is still active after PKC activation.	Santafé (2009)
	Sp-8-Br-cAMP	Increase. PKA can promote ACh release independently of PKC activation.	Santafé (2009)
Sp-8-Br-cAMP	CaC	Decrease. PKA couples PKC to ACh release.	Santafé (2009)
	PMA	None. PKA-mediated PKC activity cannot be further increased by direct PKC activation.	Santafé (2009)
H-89	PMA	None. PKA is necessary for PKC activation.	Santafé (2009)
	CaC	None. PKA is necessary for PKC activation (Table 2: CaC *per se* does not affect EPP amplitude).	Santafé (2009)

On the other hand, PKA blockade *per se* does not reduce the amount of ACh inside single vesicles in mammal NMJs. The inhibitor H-89 does not modify the amplitude or the frequency of MEPPs in the rat LAL muscle (5μM, 1h and 3h) (Santafé *et al.*, 2009) and neither in the mice diaphragm (1μM, 1h) (Gaydukov *et al.*, 2016, 2020). Interestingly, presynaptic PKA participates in the regulation of ACh loading into synaptic vesicles in mice motor synapses. In this regard, although H-89 *per se* does not affect quantal size, the PKA blockade prevents several presynaptic molecular cascades from increasing it (Gaydukov and Balezina, 2006; Gaydukov *et al.*, 2016, 2019). Additionally, the actions of PKA are not limited to the presynaptic component of the NMJ. On a longer time scale (7-8 hours), the activation of PKA largely prevents the removal of recycled nAChRs from postsynaptic membrane in mice NMJs (Martinez-Pena y Valenzuela *et al.*, 2013). This stability and quantity of postsynaptic receptors is probably controlled by postsynaptic PKA.

In conclusion, PKA at the NMJ promotes the loading of ACh inside synaptic vesicles, their release susceptibility and the number of postsynaptic nAChRs. All these findings demonstrate that PKA is one of the most important kinases that regulate the dynamics of synaptic release at the NMJ.

Muscarinic signaling and PKA at the NMJ

Classically, M_2 and M_4 mAChR subtypes are known to inhibit PKA through the $G\alpha_{i/0}$ protein pathway (Caulfield and Birdsall, 1998). At the NMJ, muscarinic receptors use PKA to modulate neurotransmission (**Table 3**) (Santafé *et al.*, 2006). For example, pirenzepine (M_1), which downregulates the endplate potential, prevents any further decrease by the PKA inhibitor H-89. Therefore, the block of M_1 results in a reduction in PKA activity (Santafé *et al.*, 2006). Under the same M_1 blockade conditions, the PKA activator Sp-Br-cAMPs is not able to enhance the release anymore (Santafé *et al.*, 2007). This indicates that PKA is linked to the M_1 signaling and that it requires an active M_1 signaling to enhance neurotransmission. The same occurs after the treatment with methoctramine (M_2), which abolishes the effect of PKA activators and inhibitors (Santafé *et al.*, 2006). Overall, it appears that both M_1 and M_2 blocking treatments uncouple PKA from the neurotransmission cascade and render it inactive and unresponsible to activation (Santafé *et al.*, 2006). Interestingly, the treatment with atropine (unspecific blocker) did not influence the PKA signaling (Santafé *et al.*, 2006). In other words, in atropine-treated preparations both H-89 and Sp-Br-cAMPs can inhibit and enhance, respectively, the synaptic release.

Besides the direct cAMP signaling, mAChRs could also modulate PKA through PKC signaling. At the adult NMJ, a previous treatment with the PKCε inhibitor $\boldsymbol{\varepsilon V_{1\text{-}2}}$ abolishes the effect of Sp-8-Br-cAMPs (Obis *et al.*, 2015b). Therefore, without nPKCε, PKA cannot be enhanced to potentiate the synaptic release. On the other hand, H-89 can still decrease neurotransmission after nPKCε inhibition, suggesting that the basal activity of PKA is still present and independent of PKCε (Obis *et al.*, 2015b). Thus, the nPKCε isoform appears to be linked to an upstream position of PKA, enhancing its activity but being less important to initiate it.

As to PKA is required or not for muscarinic signaling, there is no data showing whether a preincubation of a PKA drug affects the result of pirenzepine, methoctramine or atropine over the EPP amplitude. In this

thesis, we designed a set of the experiments to study whether muscarinic receptors need PKA to affect the phosphorylation of the release machinery.

Table 3. PKA influence over muscarinic signaling. Current knowledge from electrophysiological experiments in frog and rat muscle preparations muscarinic and PKC modulators. Data of single drug experiments has been added in the first rows to aid interpretation. Abbreviations: EPP, evoked endplate potential.

Pretreatment (1)	PKA treatment (2)	Effect on EPP amplitude (2 vs 1)	Reference
Control (physiological conditions)	H-89	Decrease. PKA promotes ACh release in basal conditions.	Santafé (2006)
	Sp-8-Br-cAMPs	Increase. PKA promotes ACh release in basal conditions.	Santafé (2006)
	Pirenzepine (M_1)	Decrease. M_1 enhances the release.	Slutsky (1999) Santafé (2003)
	Methoctramine (M_2)	Increase. M_2 inhibits the release.	Slutsky (1999) Santafé (2003)
	Atropine (M_1-M_5)	Increase. Overall muscarinic inhibition enhances the release (i.e. $M_2 > M_1$).	Santafé (2003)
Pirenzepine	H-89	None. M_1 blockade reduces PKA activity.	Santafé (2006)
	Sp-8-Br-cAMPs	None. M_1 blockade prevents further PKA activation.	Santafé (2006)
Methoctramine	H-89	None. M_2 blockade reduces PKA activity.	Santafé (2006)
	Sp-8-Br-cAMPs	None. M_2 blockade prevents further PKA activity.	Santafé (2006)
Atropine	H-89	Decrease. PKA can be inhibited after overall muscarinic inhibition.	Santafé (2006)
	Sp-8-Br-cAMPs	Increase. PKA can be enhanced after overall muscarinic inhibition.	Santafé (2006)
$\varepsilon V_{1\text{-}2}$	H-89	Decrease. PKA can be inhibited after PKCε inhibition.	Obis (2015b)
	Sp-8-Br-cAMPs	None. PKA cannot be activated after PKCε inhibition.	Obis (2015b)

4. Protein kinase C and calcium signaling

The inflow of calcium ions (Ca^{2+}) inside the nerve ending is one of the most crucial events for synaptic function. This occurs when axon potentials arrive to the nerve ending, decrease the membrane potential and open voltage-dependent calcium channels. In adult neuromuscular junctions, the muscarinic receptor M_1 subtype contributes to the entrance of calcium through the coupling with $G\alpha_{q/11}$ proteins. The high intracellular Ca^{2+} concentration modulates several calcium-sensitive proteins, but one of them stands out as the major coordinator of the cascade: the protein kinase C.

4.1. The protein kinase C

Members of the PKC family

The Ca^{2+}-dependent protein kinase is a serine/threonine kinase also known as protein kinase C or PKC. This enzyme is activated by allosteric binding to several second messengers and membrane phospholipids (Huang, 1990).

PKC isoforms are classified in accordance with their domain composition, which dictates their requirement for catalytic activation (Newton, 2010). All PKC isoforms have two regions, regulatory and catalytic, joined by a hinge region (■ Figure 6-A). In the regulatory moiety or region, the pseudosubstrate domain (PS) imitates the sequence of a PKC substrate to bind the catalytic region and inhibit kinase activity. To unfold this domain and activate the kinase, the other domains in the regulatory region need to bind the appropriate second messenger and change the molecular conformation of the enzyme. PKC isoforms are classified in three families based on their activation requirements. **Conventional** or **classical PKCs** (cPKCα, -βI, -βII and -γ) need to bind DAG and phosphatidylserine to their C1 regulatory domain and Ca^{2+} to their C2 regulatory domain to activate their kinase function. **Novel PKCs** (nPKCδ, -ε, -η and -θ) lack the Ca^{2+}-binding domain in C2 and, therefore, only require DAG and phosphatidylserine for their activation. Finally, **atypical PKCs** (aPKCζ and -ι/λ, being ι the human isozyme and λ the murine) lack the entire C2 domain and one cysteine-rich

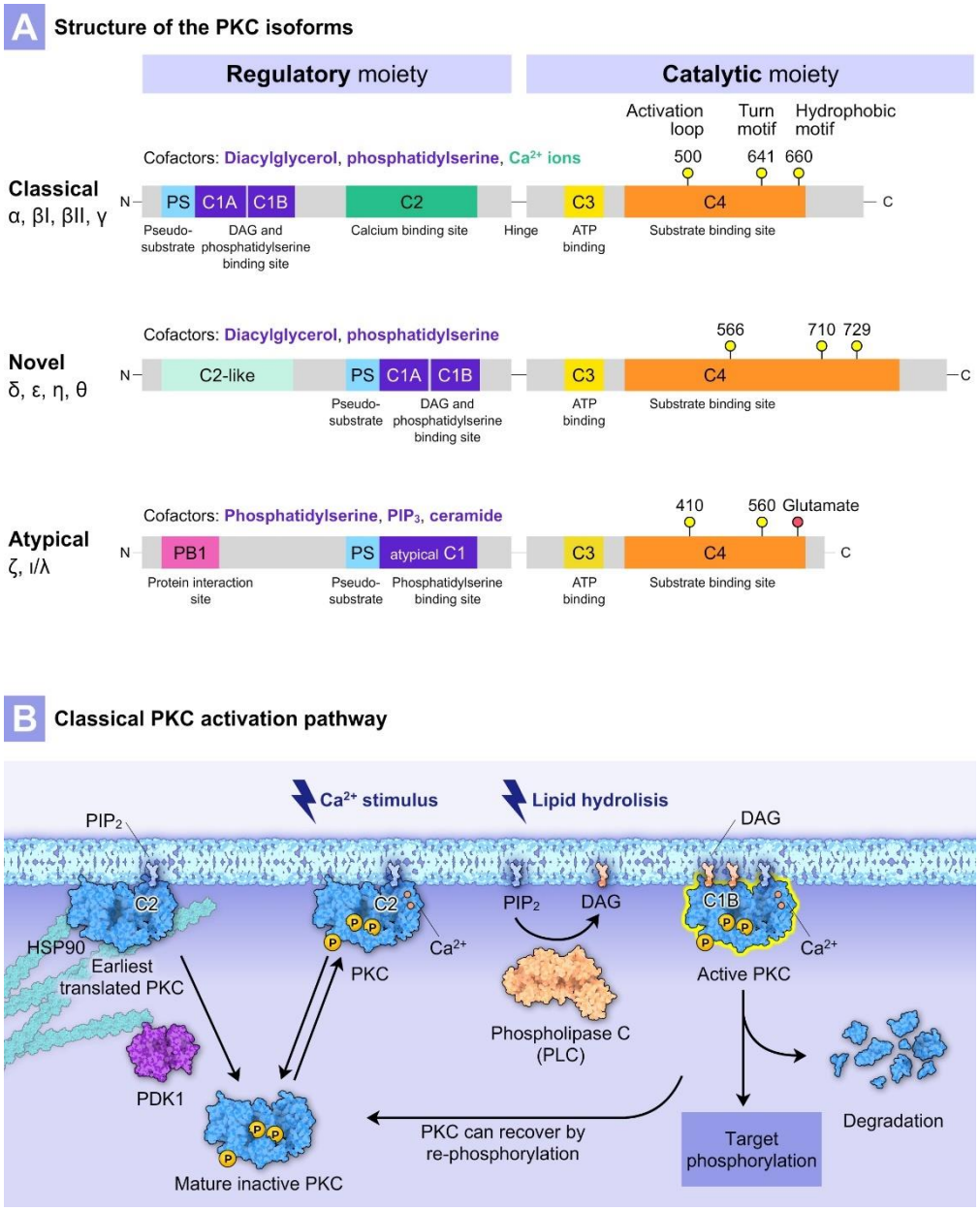

Figure 6. **The PKC superfamily.** A Protein kinase C (PKC) isoforms are classified in three families based on their activation requirements. Classical or conventional PKC (α, βI, βII and γ), novel PKC (δ, ε, η, and θ) and atypical PKC (ζ and ι/κ). The figure also depicts the pseudosubstrate region (PS) at the regulatory moiety and the priming phosphorylation sites at the catalytic moiety. B Mechanism of classical PKC activation. Newly synthesized PKC are unphosphorylated and associate with the cytoskeleton (far left). These products depend on heat shock proteins (Hsp90) to avoid degradation. The 3-phosphoinositide-dependent kinase 1 (PDK1) phosphorylates PKC on the activation loop and causes the subsequent autophosphorylations in the turn motif and the hydrophobic motif. Once mature, PKC becomes sensitive to the different stimuli that can activate it and change the cellular location. Calcium ions and diacylglycerol (DAG) promote PKC translocation to the membrane and its activity. Finally, PKC activity triggers its dephosphorylation and degradation. *Source: Adapted from Newton et al. (2008).*

loop in the C1 domain (atypical C1), thus being sensitive to lipid molecules like phosphatidylserine, phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and ceramides but not to DAG or Ca^{2+}. Phosphatidylserine and DAG are components of cellular membranes, so the binding of the C1 and C2 domains to these ligands targets PKC to the membrane (Shirai and Saito, 2002). In consistence with that, Ca^{2+} ionophores induce the translocation to the plasma membrane of green fluorescent protein fused to full-length PKCα (Almholt *et al.*, 1999) and PKCγ (Sakai *et al.*, 1997). Once activated, PKC preferentially phosphorylates a polypeptide on a residue found in close proximity to a C-terminal basic residue:

-Ser-X-Arg/Lys- - Thr-X-Arg/Lys-

Finally, after PKC has participated in the signaling cascade, it undergoes a strong turnover process known as **activity-dependent degradation** (Parker *et al.*, 1995; Lee *et al.*, 1996; Lu *et al.*, 1998; Kang *et al.*, 2000).

PKC maturation: role of PDK1

Conventional and novel PKCs are phosphorylated on three serine/threonine residues (two for atypical PKCs) over their catalytic moiety (■ Figure 6-A). This event is known as **PKC maturation** or **priming** and it is necessary for their catalytic competence and correct subcellular location in the resting state as well as represents a rate-limiting step for PKC activation (Newton, 2001; Shirai and Saito, 2002).

The earliest translation products of PKCs are unphosphorylated and remain associated with the cytoskeleton (■ Figure 6-B) (Newton, 1997). These products are open, unstable, and depend on heat shock proteins (Hsp90) to avoid degradation. The **3-phosphoinositide-dependent kinase 1** (**PDK1**) starts the first maturation step: the reversible phosphorylation of Thr^{500} (for conventional isoforms) in the activation loop, a region in the catalytic domain of PKC but outside the active site (Orr and Newton, 1994; Adams, 2003). This causes the subsequent PKC autophosphorylations on Thr^{641} in the turn motif and Ser^{660} in the hydrophobic motif (Dutil *et al.*, 1998). These Ser/Thr residues are conserved across the PKC family, suggesting that all PKC isoforms

undergo a similar maturation pathway. After this series of phosphorylations, PKC becomes sensitive to the different stimuli that can activate them and changes cellular location. For example, PKCβ isoform is initially anchored to the cytoskeleton and, after maturation, it is released into the cytosol (Edwards and Newton, 1997; Perkins *et al.*, 2001).

Although PKC maturation is constitutive, some PKC isoforms exist in a non-phosphorylated or hypo-phosphorylated state, and cellular stimulation can induce their phosphorylation and activation (Zhou *et al.*, 2003; Wang *et al.*, 2007; Osto *et al.*, 2008). At the neuromuscular junction, we found that both presynaptic stimulus and muscle contraction enhance the phosphorylation of cPKCβI (Hurtado *et al.*, 2017a).

PDK1 is a master kinase that modulates as many as 23 other protein kinases, including PKC (Toker, 2003; Mora *et al.*, 2004; Bayascas, 2010; Pearce *et al.*, 2010). At the skeletal muscle, PDK1 is exclusively expressed at presynaptic nerve terminals of both rodent and *Drosophila* NMJs (Cheng *et al.*, 2011; Hurtado *et al.*, 2017b). PDK1 is autophosphorylated on multiple serine residues: Ser^{25}, Ser^{241}, Ser^{393}, Ser^{396} and Ser^{410} (■ Figure 7-A). Among them, Ser^{241} is the only that, when mutated, abolishes PDK1 activity (Casamayor *et al.*, 1999). These phosphorylations were firstly described as constitute but subsequent studies identified pathways that modulate PDK1 phosphorylation (Scheid *et al.*, 2005; Kim *et al.*, 2013; Toricelli *et al.*, 2017). Our laboratory determined that PDK1 Ser^{241} phosphorylation is not affected by presynaptic activity or muscle contraction (Hurtado *et al.*, 2017b). However, presynaptic activity translocates phospho-PDK1 from the cytosol to the membrane fraction (Hurtado *et al.*, 2017b). PDK1 needs to be in the plasma membrane to interact with PKC and phosphorylate it (Chou *et al.*, 1998; Dutil *et al.*, 1998; Le Good *et al.*, 1998; Balendran *et al.*, 2000) and, at the NMJ, the recruitment of PDK1 to the membrane coincides with an enhancement of phospho-PKCβI (Hurtado *et al.*, 2017b). Studying the upstream kinase PDK1 could help us understand how muscarinic pathways confluence over the second-messenger kinase PKC, coordinating its maturation (■ Figure 2-C). Thus, we investigated the role of PDK1 in muscarinic signaling at the NMJ.

PKC location and scaffolding: role of RACK1

The cellular location of PKC is regulated by anchoring proteins like the **receptors for activated C-kinase** (**RACKs**) and AKAPs. These scaffolds poise PKC isoforms to certain intracellular locations to respond rapidly to second messengers and facilitate access to their substrates (Mochly-Rosen, 1995).

RACK has seven equal protein-protein interaction motifs (■ Figure 7-B) that allow it to interact with at least two proteins at a time (Schechtman and Mochly-Rosen, 2001). Several regions in the sequence of PKCs contain RACK-binding sites, mainly **C2** but also C1A and C1B (Ron *et al.*, 1994). These sites are specific to the PKC signaling, and RACK does not bind to other kinases like the PKA RII subunit or Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) (Ron et al., 1994). However, the C2 domain can be found in enzymes like **PLC**, which RACK can anchor close to PKC. By this mechanism, the presence of **RACKs increases PKC phosphorylation by several folds** although not being PKC substrates (Schechtman and Mochly-Rosen, 2001).

RACKs only bind the active conformation of PKC (■ Figure 7-C) (Mochly-Rosen, 1995; Schechtman and Mochly-Rosen, 2001). This is because PKC needs to bind to second messengers and open its structure to expose the RACK binding site. Indeed, PKC and RACK only co-immunoprecipitate after PMA stimulation or the activation of a receptor coupled to DAG generation (Csukai *et al.*, 1997).

On the other hand, RACKs are isoform-selective. Some members of the RACK family are **RACK1**, selective for the conventional PKCβ (Ron *et al.*, 1994), and **RACK2**, selective for the novel PKCε (■ Figure 7-D) (Csukai *et al.*, 1997). **RACK1** enhances PKC activity by binding PLCγ. The RACK1-PLCγ interaction increases after PLCγ phosphorylation and after cells have been activated with epidermal growth factor (Disatnik *et al.*, 1994). Thus, RACK1 provides an efficient mean of receptor-induced activation of PKC (Schechtman and Mochly-Rosen, 2001). On the other hand, RACK scaffolds also bring together a PKC to its substrates. For example, one of the earliest reports of PKCε interaction with a protein of vesicle trafficking was through **RACK2**

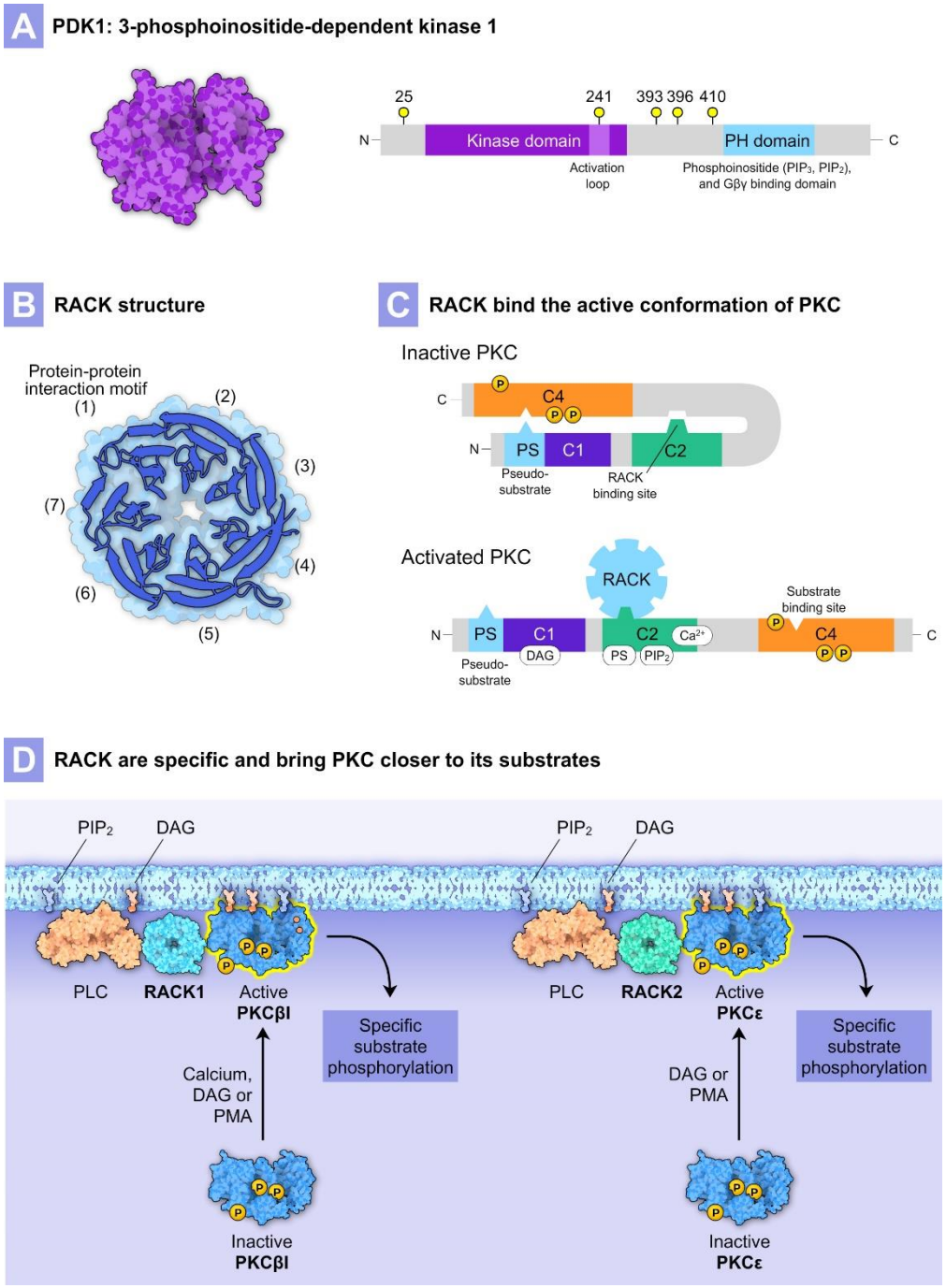

Figure 7. **Proteins that modulate PKC.** A The 3-phosphoinositide-dependent kinase 1 (PDK1) is a master kinase. It has two regions: the kinase domain and a pleckstrin-homology (PH) domain. The PH domain binds phosphoinositides (PIP_3, PIP_2) and regulates the kinase activity by interacting with the protein Gβγ subunit and PKC. PDK1 is autophosphorylated on multiple serine residues: Ser^{25}, Ser^{241}, Ser^{393}, Ser^{396} and Ser^{410}. Among them, Ser^{241} at the activation loop is required for PDK1 activity. B The receptors for activated C-kinase (RACKs) are a family of PKC scaffolds. RACKs have seven equal protein-protein interaction motifs to bind PKC and other molecules like phospholipase C (PLC). C RACKs only anchor active PKCs, because PKC requires binding to second messengers to open its structure and expose the RACK binding sites. D Each PKC isoform has a specific RACK. PKC positioning is central to respond efficiently to second messengers and to have ready access to substrates. *Source: Own elaboration.*

(Csukai *et al.*, 1997). RACK2, also called β'COP or εRACK, is part of the coat complex (COP I), which associates with vesicles from the Golgi apparatus to the endoplasmic reticulum. This explains why PKCε is recruited to Golgi membranes after activation in cardiac myocytes (Csukai *et al.*, 1997). Therefore, peptides based on the RACK binding site from PKC can disrupt the activity of a specific PKC isoform (Johnson *et al.*, 1996; Newton, 1997; Mochly-Rosen and Gordon, 1998; Stebbins and Mochly-Rosen, 2001). As explained in the Methods section, in this thesis we used RACK-inhibitory peptides to study the action of PKCβI and PKCε.

Finally, two other mechanisms should be considered to understand PKC location and movement between the cytosol and membrane. On the one hand, newly synthetized PKC *per se* can bind to the cytoskeleton and stay associated with the particulate fraction (membranes) (Newton, 1997). For example, PKCβII–but not PKCβI–binds to actin through the C-terminus (Blobe *et al.*, 1996) and PKCε binds to actin through a sequence between the C1A-C1B domains (Prekeris *et al.*, 1996) and to cytoskeletal components through the hinge and pseudosubstrate regions (Lehel *et al.*, 1995). On the other hand, other scaffolds like AKAP79–AKAP150 rodent homologue–can also target PKC isoforms alongside other enzymes like PKA and calcineurin (Klauck *et al.*, 1996; Perkins *et al.*, 2001).

4.2. PKC at the neuromuscular junction

PKC location at the neuromuscular junction

Since each PKC isoform requires a particular combination of second messengers, the presence of multiple isoforms in the same cell provides a fine mechanism of isotype-specific regulation, where each isozyme may have functional specificity (Nakano *et al.*, 1992; Shirai and Saito, 2002). At the NMJ, PKC isoforms are differently expressed in the presynaptic terminal, the postsynaptic myocyte and Schwann cells (■ Figure 8).

The conventional **PKCα** isoform is the major PKC isoform present in muscle and can be found in all three cellular components of the NMJ (Nakano *et al.*, 1992; Besalduch *et al.*, 2010). The first reports of **PKCβ** immunoreactivity

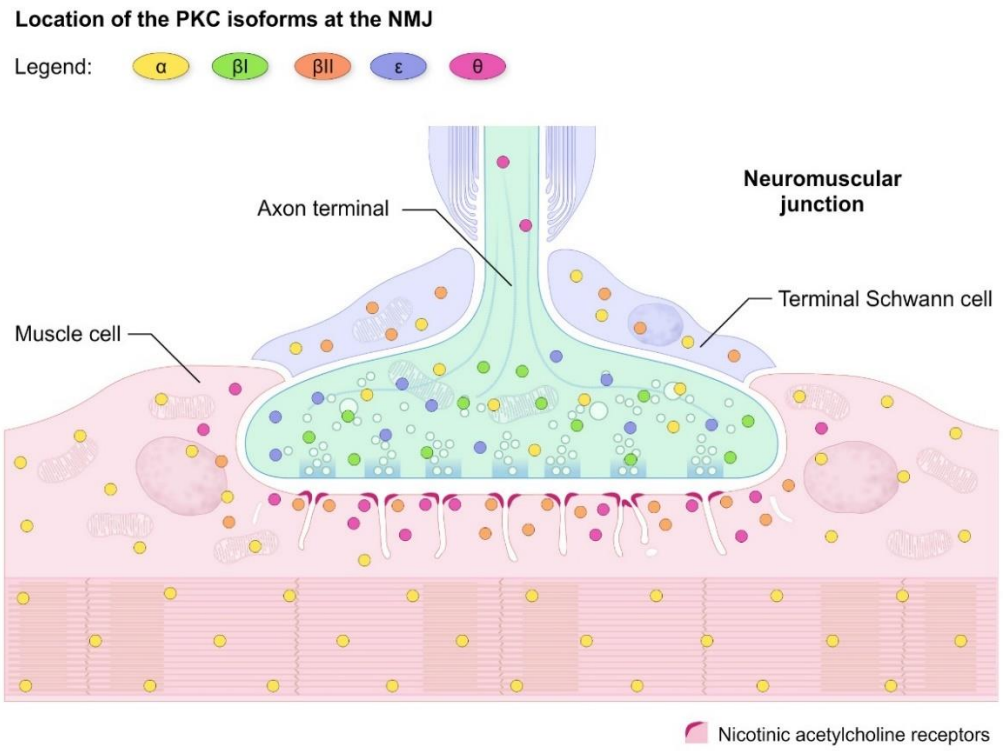

Figure 8. **Current knowledge of the location of PKC isoforms at the NMJ.** The classical PKCα isoform (yellow) can be found in all three components of the neuromuscular junction. The PKCβI (green) is exclusively expressed in the presynaptic nerve terminals, whereas the PKCβII isoform (orange) is concentrated at the postsynaptic site of NMJs and Schwann cells, but not within the nerve terminal. The novel PKCε (blue) is exclusively expressed in the nerve terminal of NMJs. Finally, the PKCθ isoform (magenta) is mainly found postsynaptically and it can be detected in the sarcolemma of skeletal muscle and in the axon of some synapses. *Source: own elaboration.*

(without discerning between PKCβI and PKCβII) were consistently found in the presynaptic terminals of NMJs from several rat muscles (Hietanen *et al.*, 1990; Arakawa *et al.*, 1993). Afterwards, Perkins *et al.*, 2001 found PKCβ in both pre- and postsynaptic regions of the NMJ. They observed a background of diffuse cytosolic staining with two stronger cytoskeleton-associated bands, one in the presynaptic region and the other co-distributing with nAChRs. Later, our laboratory discerned PKCβI and βII isoforms and found that **cPKCβI** is exclusively expressed in the presynaptic nerve terminal (Besalduch *et al.*, 2010). In concordance, phosphorylated PKCβI is also exclusively found at the nerve terminals of NMJ from mice in development (Garcia *et al.*, 2019). On the other hand, **cPKCβII** is concentrated at the postsynaptic site of NMJs, partially overlies with Schwann cells, but it is not detected within the nerve terminal (Besalduch *et al.*, 2010).

The novel **nPKCε** isoform is exclusively expressed in the nerve terminal of NMJs from rat LAL muscles (Obis *et al.*, 2015) and mice semitendinous muscles (Maeno-Hikichi *et al.*, 2011). Triple staining of NMJ cross-sections show that PKCε label is punctate over postsynaptic AChR gutters, colocalizes with the presynaptic terminal and is absent in Schwann cells and the myocyte (Obis *et al.*, 2015a). On the other hand, **nPKCθ** staining at the NMJ persists after denervation, showing that the enzyme is distributed postsynaptically (Hilgenberg and Miles, 1995). This isoform is found associated with the sarcolemma of skeletal muscle, enhanced at the neuromuscular junction and, in some synapses, it could be detected in the axon of motoneurons (Hilgenberg and Miles, 1995; Lanuza *et al.*, 2010).

Finally, **PDK1** is exclusive of the presynaptic terminal of NMJs, being detected in pre-terminal axons and concentrated at the presynaptic area over the postsynaptic gutters (Hurtado *et al.*, 2017b).

In this thesis, we have worked with PKCα, -βI, and -ε; but special effort has been made to study the conventional PKCβI and the novel PKCε isoforms, due to their presynaptic location and their role in neurotransmitter release (Lanuza *et al.*, 2014; Obis, *et al.*, 2015; Simó *et al.*, 2019).

PKC modulation of acetylcholine release

Several agonists and antagonists have been used to study the role of PKC on ACh release at the NMJ. Phorbol 12-myristate 13-acetate (**PMA**) is a classic activator that mimics DAG and induces PKC translocation to the membrane (Raffaniello and Raufman, 1994; Ohmori *et al.*, 1998). Calphostin C (**CaC**) is an inhibitor that interacts with the regulatory diacylglycerol binding site and phorbol ester binding site of PKC, blocking both conventional and novel PKC isoforms (Kobayashi *et al.*, 1989).

PKC promotes ACh release at the NMJ, but requires several conditions to bring out its effect, something also referred as "**couple**" its activity to neurotransmission. This is because, under physiological conditions, the neuromuscular transmission does not have a PKC-dependent component that can be inhibited with calphostin C (Santafé *et al.*, 2006).

However, PKC activity at the NMJ flourishes after any event that modifies its second messengers or its upstream membrane receptors (Table 4). Treatments that enhance PKC second messengers, like high Ca^{2+} concentration or synaptic stimulation, activate PKC and make CaC able to downregulate the neurotransmitter release (Santafé *et al.*, 2007; Besalduch *et al.*, 2010). PKC can also be coupled by muscarinic receptors, which has been described in the following section (Table 5).

Nerve-induced stimulus (1 Hz, 30 minutes) triggers multiple mechanisms at the NMJ: the direct calcium inflow into the presynaptic terminal, the cholinergic signaling and neurotrophic signaling, all of them connecting to PKC (Slutsky *et al.*, 1999; Besalduch *et al.*, 2010; Hurtado *et al.*, 2017a). Therefore, synaptic activity recruits PKC to cooperate with neurotransmission. All this evidence suggests that PKC may be necessary to maintain acetylcholine release once the process is activated.

PKC coupling to ACh release also occurs after **PKA stimulation.** For example, the PKA agonist Sp-8-Br-cAMP enhances neurotransmission and makes CaC able to decrease ACh release (Santafé *et al.*, 2009). In fact, PKC activity depends on PKA, and even the potent PKC activator PMA cannot increase neurotransmission if PKA is previously inhibited with H-89. Interestingly, it should be noted that PKA does not depend on PKC, so activating or blocking PKC does not affect PKA action over ACh release (Santafé *et al.*, 2009).

Specific inhibitor peptides have been designed to study PKC isoforms (Mochly-Rosen and Gordon, 1998; Budas *et al.*, 2007). In this thesis we have studied the presynaptic cPKCβI and nPKCε isoforms, respectively, with the $\beta IV_{5\text{-}3}$ peptide (**$\beta IV_{5\text{-}3}$**) (Liu *et al.*, 1999; Zhang *et al.*, 2015) and the $\varepsilon V_{1\text{-}2}$ peptide (**$\varepsilon V_{1\text{-}2}$**) (Johnson *et al.*, 1996). These peptides derive from the sequence that a specific PKC isoform uses to interact with its RACK scaffold. Therefore, the peptide competes with just one PKC isoform and disrupts its cellular targeting and activity. The cPKCβI isoform directly promotes the activity-dependent neurotransmitter release at the NMJ. In particular, 1 Hz stimulation triggers a PKC activity with a cPKCβI-component that can be detected and blocked with $\beta IV_{5\text{-}3}$ (Hurtado *et al.*, 2017a). On the other hand, nPKCε does not have

a direct influence on acetylcholine release: $\varepsilon V_{1\text{-}2}$ *per se* does not change the EPP size. Even other treatments that would normally activate PKC, like high Ca^{2+} or 1 Hz stimulation, do not trigger a PKCε activity that can be detected with $\varepsilon V_{1\text{-}2}$ (Obis *et al.*, 2015b). However, nPKCε influences other PKC isoforms. For example, when nPKCε is blocked, PMA is not able to increase the release anymore. Similarly, if $\varepsilon V_{1\text{-}2}$ is present in the media, neither a high concentration of Ca^{2+} nor 1 Hz synaptic stimulus can recruit PKC, and general PKC activity cannot be inhibited with CaC anymore (Obis *et al.*, 2015b). This indicates that nPKCε facilitates the activation of the other PKC isoforms.

The functional data from electrophysiology can be complemented with molecular studies determining which targets does PKC phosphorylate at the NMJ. PKC has been involved in the regulation of ligand-gated ion channels (Swope *et al.*, 1999; Nelson *et al.*, 2003). The SNARE regulator Munc18-1 (Ser^{313}) is phosphorylated by PKCβI and PKCε at the NMJ (Simó *et al.*, 2018). On the other hand, the synaptosomal-associated protein 25 (SNAP-25) is controlled by PKCε but not PKCβI (Simó *et al.*, 2019). PKCε also phosphorylates the PKC substrate Myristoylated Alanine-Rich protein Kinase C Substrate (MARCKS) (Obis *et al.*, 2015a). Altogether, these results suggest that the presynaptic cPKCβI and PKCε are good candidates to transduce mAChR signaling.

Table 4. PKC coupling to the release of the adult NMJ. Current knowledge from electrophysiological experiments in rat muscle preparations (Santafé *et al.*, 2009; 2006; 2007; Obis *et al.*, 2015b; Besalduch et al., 2010; Hurtado et al., 2017a) testing PKC modulators. Abbreviations: EPP, evoked endplate potential.

Pretreatment (1)	PKC treatment (2)	Effect on EPP amplitude (2 vs 1)	Reference
Control (physiological conditions)	CaC	None. PKC is not coupled to ACh release in basal conditions.	Santafé (2006) Santafé (2007)
	PMA	Increase. Phorbols promote ACh release.	Santafé (2006) Santafé (2007)
	High Ca^{2+}	Increase. Calcium potentiates ACh release.	Santafé (2007)
	High Mg^{2+}	Decrease. Magnesium, which competes with calcium, blocks ACh release.	Santafé (2007)
	1 Hz	None. Synaptic activity at 1 Hz does not induce facilitation or depression.	Besalduch (2010) Obis (2015b)
CaC	PMA	None. CaC antagonizes the effect of PMA	Obis (2015b)
PMA	CaC	None. After PKC activation with PMA, CaC does not reduce ACh release	Obis (2015b)
1 Hz	CaC	Decrease. Synaptic activity couples PKC to ACh release.	Besalduch (2010)
	$\beta IV_{5\text{-}3}$	Decrease. Synaptic activity couples PKCβI to ACh release.	Hurtado (2017a)
	$\varepsilon V_{1\text{-}2}$	None. Synaptic activity does not couple PKCε to ACh release.	Obis (2015b)
High Ca^{2+}	PMA	None. PMA cannot increase further the release.	Santafé (2007)
	CaC	Decrease. PKC partly mediates the increased release in high Ca^{2+}.	Santafé (2007) Besalduch (2010)
High Mg^{2+}	PMA	None. Magnesium, which competes with calcium, prevents PKC activation with PMA.	Santafé (2007)
	CaC	None. CaC has no effect on the low Ca^{2+} entry produced in high Mg^{2+}.	Obis (2015b)
$\varepsilon V_{1\text{-}2}$	PMA	None. PKCε is essential to couple PKC (isoforms) to ACh release.	Obis (2015b)
Sp-8-Br-cAMP	CaC	Decrease. PKA activation couples PKC to ACh release.	Santafé (2009)
	PMA	None. PKA-mediated PKC activity cannot be further increased by PKC activation.	Santafé (2009)
H-89	CaC	None. When PKA is blocked, the release cannot be inhibited by CaC.	Santafé (2009)
	PMA	None. Without PKA, PKC cannot be coupled to ACh release.	Santafé (2009)

Muscarinic signaling and PKC at the NMJ

Classically, M_1, M_3 and M_5 mAChR subtypes are known to activate PKC through the $G\alpha_{q/11}$ protein pathway (Caulfield and Birdsall, 1998). At the NMJ, **muscarinic imbalance is one of the conditions that couple PKC** activity to neurotransmitter release (**Table 5**) (Santafé *et al.*, 2006, 2007). For example, the treatment with pirenzepine (M_1), which downregulates the endplate potential, allows the PKC inhibitor CaC to further reduce the amplitude of synaptic impulses. Under the same M_1 blockade conditions, the PKC activator PMA can still increase the release (Santafé *et al.*, 2007). This indicates that the PKC pathway is downstream of the signaling and can be recruited independently of the M_1 pathway. The same occurs after the treatment with methoctramine (M_2) or atropine (unspecific) at the NMJ. Both treatments increase the synaptic release and couple PKC and allow CaC to inhibit the synaptic release (Santafé *et al.*, 2006).

On the other hand, PKC is essential for muscarinic signaling. This was first demonstrated with a **preincubation of CaC**, which abolishes the effect of pirenzepine, methoctramine and atropine over the EPP amplitude (Santafé *et al.*, 2006). Afterwards, our laboratory proceeded to study the specific blockade of nPKCε isoform translocation. The treatment with **εV$_{1-2}$** abolished again the effect of any following muscarinic blocker (Obis *et al.*, 2015b). This demonstrated that nPKCε is required for the M_1 and M_2 mAChR signaling. Unlike CaC, muscarinic imbalance does not enhance the effect of PKCε inhibition. This indicates that after any muscarinic blockade, the neurotransmission cannot be affected by the nPKCε blockade. Thus, the nPKCε isoform appears to be in an upstream position of the muscarinic pathway, allowing the signaling at the first steps and being less needed once stablished (Obis *et al.*, 2015b).

Besides their classic G protein pathway, mAChRs also modulate PKC through the concentration of calcium ions in the presynaptic terminal. At the adult NMJ, mAChRs are functionally linked to voltage-dependent calcium channels (Santafé *et al.*, 2003). Therefore, experiments modifying the calcium pathway reveal changes in muscarinic signaling. For example, a low concen-

Table 5. PKC influence over muscarinic signaling. Current knowledge from electrophysiological experiments combining muscarinic and PKC modulators. Data of single drug tests have been added in the first rows to aid interpretation. Abbreviations: EPP, evoked endplate potential.

Pretreatment (1)	Muscarinic treatment (2)	Effect on EPP amplitude (2 vs 1)	Reference
Control (physiological conditions)	CaC	None. PKC is not coupled to ACh release in basal conditions.	Santafé (2006) Santafé (2007)
	PMA	Increase. Phorbols promote ACh release.	Santafé (2006) Santafé (2007)
	Pirenzepine (M_1)	Decrease. M_1 enhances the release.	Slutsky (1999) Santafé (2003)
	Methoctramine (M_2)	Increase. M_2 inhibits the release.	Slutsky (1999) Santafé (2003)
	Atropine (M_1-M_5)	Increase. Overall muscarinic inhibition enhances the release (i.e. $M_2 > M_1$).	Santafé (2003)
Pirenzepine, Methoctramine or Atropine	CaC	Decrease. Any muscarinic imbalance couples PKC to the release	Santafé (2006)
	PMA	Increase. PKC can be further activated after any muscarinic imbalance.	Santafé (2006)
CaC	Pirenzepine	None. Without PKC, M_1 cannot enhance the release.	Santafé (2006)
	Methoctramine	None. Without PKC, M_2 cannot inhibit the release.	Santafé (2006)
	Atropine	None. Without PKC, overall muscarinic inhibition cannot modulate the release.	Santafé (2006)
$\varepsilon V_{1\text{-}2}$	Pirenzepine	None. M_1 requires PKCε.	Obis (2015b)
	Methoctramine	None. M_2 requires PKCε.	Obis (2015b)
	Atropine	None. Overall muscarinic signaling requires PKCε to inhibit the release.	Obis (2015b)
Pirenzepine, Methoctramine or Atropine	$\varepsilon V_{1\text{-}2}$	None. After muscarinic modulation, PKCε does not enhance the release.	Obis (2015b)
High Ca^{2+}	Pirenzepine	Decrease. External calcium does not affect the M_1 outcome.	Santafé (2007)
	Methoctramine	Increase. External calcium does not affect the M_2 outcome.	Santafé (2007)
	Atropine	Increase. External calcium does not affect the muscarinic outcome.	Santafé (2007)
Low Ca^{2+}	Pirenzepine	Decrease. M_1 can still enhance EPP after low Ca^{2+} inhibition.	Santafé (2007)
	Methoctramine	Decrease. M_2 shifts to enhance EPP after low Ca^{2+} inhibition.	Santafé (2007)
	Atropine	Decrease. Overall muscarinic signaling shifts to enhance EPP after low Ca^{2+} inhibition.	Santafé (2007)
High Mg^{2+}	Pirenzepine	Decrease. M_1 can still enhance EPP after high Mg^{2+} inhibition.	Santafé (2007)
	Methoctramine	Decrease. M_2 shifts to enhance EPP after high Mg^{2+} inhibition.	Santafé (2007)
	Atropine	Decrease. Overall muscarinic signaling shifts to enhance EPP after high Mg^{2+} inhibition.	Santafé (2007)
High Mg^{2+} + Methoctramine	CaC	None. With high Mg^{2+}, M_2 imbalance does not promote PKC activity.	Santafé (2007)

-tration of Ca^{2+} or a **high concentration of Mg^{2+}**, which competes with Ca^{2+} and reduces the release, induces pirenzepine, methoctramine and atropine to reduce the EPP potential (Santafé *et al.*, 2007). This represents a remarkable finding for both M_1 and M_2 signaling. First, the results show a change in M_2 signaling, now potentiating the release in low Ca^{2+} media and high Mg^{2+} media (Santafé *et al.*, 2007). Further tests indicated that methoctramine was not promoting PKC activity in those conditions anymore (CaC had no effect after Mg^{2+} + methoctramine) (Santafé *et al.*, 2007). On the other hand, it is surprising that M_1 can still enhance neurotransmitter release after a Ca^{2+} signaling blockade. In the present thesis, we analyzed the potential link of M_1 mAChR with the Ca^{2+}-independent PKCε and the kinase PKA.

Altogether, these results offer solid evidence of functional crosstalk between both M_1 and M_2 mAChRs and PKC at the NMJ. Therefore, in this thesis we further explored this interesting feature by obtaining molecular data about maturation, membrane translocation and activity of the presynaptic PKCβI and PKCε isoforms and their associated kinases and targets and how they respond to muscarinic blockade.

5. Molecules of the release machinery

The synaptic vesicles interact with the neural membrane thanks to a set of proteins referred as the **release machinery**, which sense calcium and regulate membrane fusion.

The most fundamental release machinery is the **SNARE pin** (Soluble NSF Attachment Protein Receptor) or **core**, formed by three proteins: synaptobrevin, syntaxin and SNAP-25 (synaptosomal-associated protein of 25 kDa)(Südhof, 1995). The second universally required component of the release machinery are the **SM proteins** (Sec1/Munc18-like proteins), which play a complementary role in membrane fusion (Südhof and Rothman, 2009). These proteins are equally important as SNAREs and fusion *in vivo* cannot take place without them (Verhage *et al.*, 2000). Finally, the release machinery has plenty of **regulatory members** that embed the SM/SNARE fusion machinery into the physiological context (Südhof and Rothman, 2009).

5.1. SNARE proteins

Each component of the SNARE core has a specific location: **Synaptobrevin** is anchored to the membrane of the vesicle (hence classified as a 'v-SNARE'), **Syntaxin-1** is anchored to the target neural membrane ('t-SNARE') and **SNAP-25** is a soluble factor anchored to the target membrane through palmitoyl side chains (Söllner *et al.*, 1993b; Snyder *et al.*, 2006).

The SNARE superfamily is very diverse, with only a 25% of protein sequence identity (Südhof and Rothman, 2009). However, all members share at least one SNARE motif (■ Figure 9-A), responsible for self-associating between proteins and creating a **four-helix bundle** complex. Most SNARE proteins contain a single SNARE motif and are anchored to the membrane by a transmembrane domain (TMD) at their C-terminal tail. The SNAP-class of SNAREs is particular in which contains two motifs and is anchored by palmitoylation.

SNARE complex formation and neurotransmitter release

At first, individual SNARE proteins are unfolded and expose their α-helix domain containing the SNARE motif to the media. When SNARE motifs become close to each other, they spontaneously assemble into an exceptionally stable **four-helix bundle** (Sutton *et al.*, 1998), which unites the vesicle and the target membrane. This assembly is controlled by SM proteins like Munc18-1, which will be described in the following section. The first interaction is between Syntaxin and SNAP-25 and it is a rate-limiting step in SNARE complex assembly (Fasshauer and Margittai, 2004). In turn, this provides a high affinity binding site for the vesicular Synaptobrevin. The union of the three molecules between separate membranes is known as the **trans-SNARE complex** or **SNARE pin** (■ Figure 9-B). As the helices zipper up, they force the membranes closely together and catalyze their fusion. Usually, neurotransmitter release does not require the complete fusion of the vesicle but just a transient opening of the vesicle's lumen to the outside. This mechanism is known as kiss-and-run, and after part of the acetylcholine content has escaped through the pore, the vesicle is then internalized by endocytosis (Hughes *et al.*, 2006). However, this kiss-and-run mechanism has been investigated at CNS nerve terminals that are highly active and may not be as prominent at the majority of neuromuscular junctions that fire at a different rate (Hughes *et al.*, 2006). When vesicle fusion is fully complete, the assembled SNARE pin is left in the merged membrane. This complex is known as '**cis-SNARE complex**'. Finally, an ATPase will unfold it and return it to the initial state for the next vesicle cycle (Südhof and Rothman, 2009). This ATP-dependent mechanism is described in the following text.

Synaptobrevin

Synaptobrevin, also called vesicle-associated membrane protein (VAMP), is a small integral membrane protein that, as its name implies, is anchored to synaptic vesicles. Two isoforms are known: Synaptobrevin-1 and -2, being the latter the most well-known. Synaptobrevin-2 catalyzes fusion reactions and stabilizes fusion intermediates, but it is not absolutely required for synaptic fusion (Schoch *et al.*, 2001). This is because in mice lacking the gene

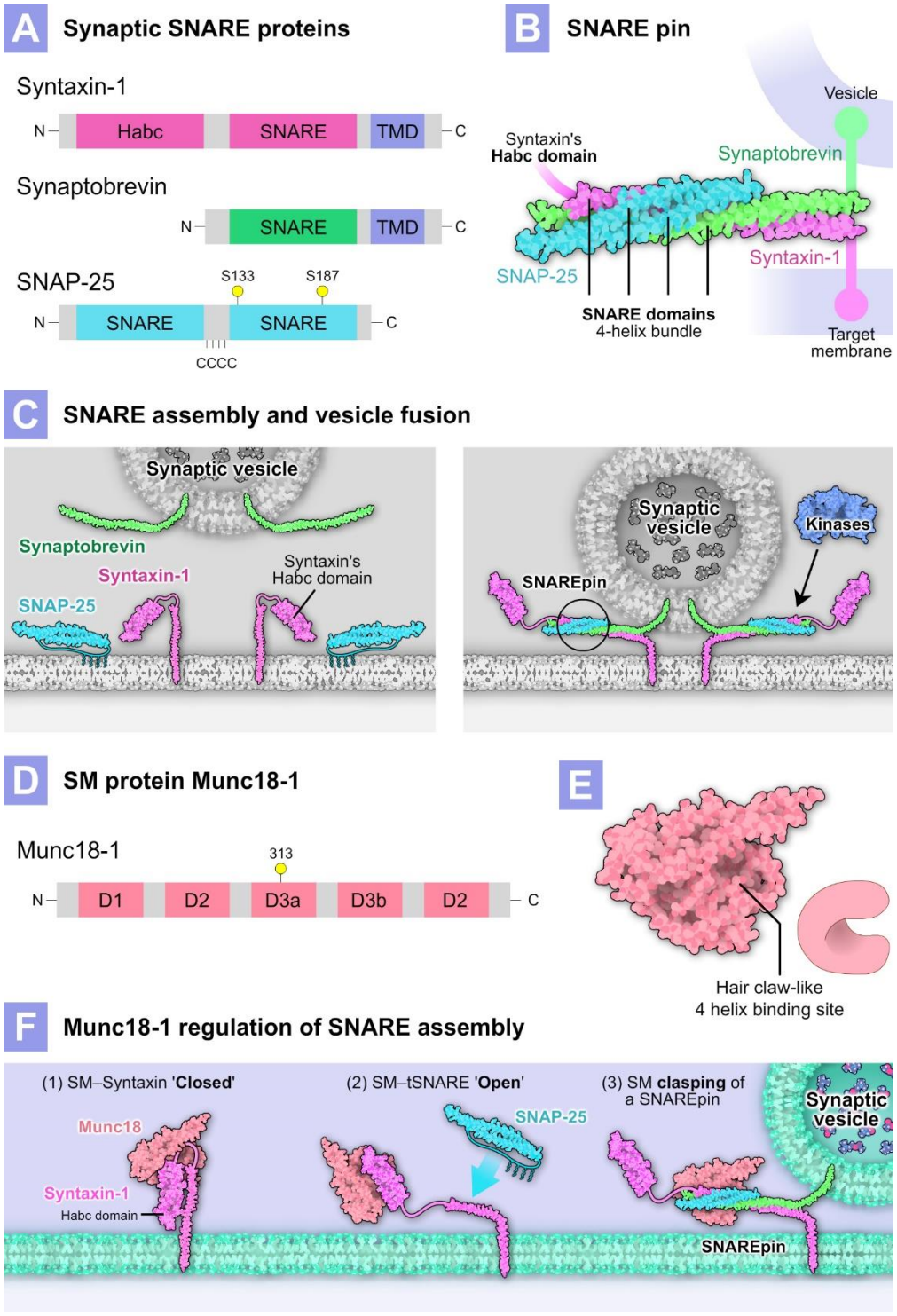

Figure 9. **SNARE and SM proteins.** A Sequence of Synaptobrevin (green), Syntaxin-1 (magenta) and SNAP-25 (blue). Syntaxin-1 and Synaptobrevin anchor to membranes with a transmembrane domain (TMD) whereas SNAP-25 through palmitoylated cysteines (C). B SNAREs spontaneously assemble into an exceptionally stable 4-helix bundle called SNARE pin. C The assembly of SNARE complex links vesicle and target membranes. Once assembled, kinases can phosphorylate it and optimize fusion. D Sequence of the SM protein Munc18-1. E Munc18-1 'hair claw'-shaped structure binds 4-helix bundles. F Munc18-1 has three ways of binding SNAREs. (1) It can hold Syntaxin-1 in a "closed" conformation, binding the Habc domain and SNARE motif. (2) To promote exocytosis, Munc18-1 binds the N-terminal region of Syntaxin, acquiring an "open" conformation. (3) Munc18-1 can bind the 4-helix bundle and mechanically enhance exocytosis. *Source: own elaboration*

Synaptobrevin-2 both spontaneous and Ca^{2+}-triggered vesicle fusion are decreased 10- and 100-fold but not totally abolished (Schoch *et al.*, 2001). This molecule is phosphorylated by the kinases CaMKII and casein kinase II (Nielander *et al.*, 1995). Lastly, Synaptobrevin also participates in slow, clathrin-dependent endocytosis, retrieving exocytosed vesicles for recycling. This mechanism is critical for the maintenance of synaptic transmission and the normal structure of nerve terminals (Zhang *et al.*, 2013).

Syntaxin-1

The SNARE member **Syntaxin-1** has three domains: the N-terminal Habc domain, a SNARE motif and a C-terminal transmembrane region. Of particular interest is the Habc domain, comprised of three α-helices. When Syntaxin-1 is not participating in a SNARE complex, the Habc domain (3 helices) folds back with the SNARE motif (1 helix), creating a 4-helix bundle known as the **'closed' conformation** of Syntaxin-1 (Dulubova *et al.*, 1999). As described below, the Syntaxin-1 closed conformation is clamped by SM proteins to avoid the formation of SNARE pins (Südhof, 1995; Wu *et al.*, 1998).

SNAP-25

The third SNARE core component is **SNAP-25** (Oyler, 1989). This molecule has two SNARE motifs connected through a loop, which contains cysteine residues palmitoylated for membrane targeting (Gonzalo *et al.*, 1999). These two SNARE motifs interact with the other SNARE domains from Syntaxin-1 and Synaptobrevin to form a four-helix SNARE pin. SNAP-25 is essential for exocytosis. In mice lacking SNAP-25, calcium-triggered exocytosis is totally abolished (Sørensen *et al.*, 2003). On the other hand, SNAP-25 participates in the organization of vesicles, being necessary for the filling of the pool of primed vesicles for release (Sørensen *et al.*, 2003). SNAP-25 is a substrate of PKC at Ser^{187} (Shimazaki *et al.*, 1996; Kataoka *et al.*, 2000) and PKA at Thr^{138} (Risinger and Bennett, 1999; Nagy *et al.*, 2004).

PKC phosphorylation of SNAP-25 is low in resting cells, but can be induced through PKC-activating treatments like Ca^{2+} increase (Nagy *et al.*, 2002); phorbol esters and nerve growth factor in PC12 cells (Shimazaki *et al.*,

1996; Kataoka *et al.*, 2000); and long-term potentiation (LTP) in hippocampal neurons (Genoud *et al.*, 1999). PKC phosphorylation of SNAP-25 promotes the refilling after the pools have been emptied (Nagy *et al.*, 2002). In particular, the unphosphorylatable S187A mutants inhibits the rate of vesicle refilling. On the other hand, *in vitro* phosphomimetic S187E mutations of SNAP-25 increase the exocytosis of the highly Ca^{2+}sensitive pool of vesicles and increase the binding of recombinant SNAP-25 with syntaxin (Yang *et al.*, 2007).

PKA phosphorylation of SNAP-25 produces confusing results between exocytosis at the cellular level and SNARE complex formation at the molecular level. Constitutive PKA activity on SNAP-25 enhances Ca^{2+}-dependent exocytosis and maintains the releasable and primed vesicle pools in chromaffin cells (Nagy *et al.*, 2004). Chromaffin cells have two characterized releasable and primed vesicle pools: the slowly releasable pool (SRP) and the ready-releasable pool (RRP), which are released in slow and fast exocytotic burst upon stimulation (Hirling and Scheller, 1996; Leenders and Sheng, 2005). PKA maintains those pools because the SNAP-25 mutant T138A causes a 2-fold decrease in fast and slow exocytotic burst components. However, the mutant T138D, which mimics a phosphorylated Thr^{138}, does not modify secretion significantly. Thus, tonic PKA activity on SNAP-25 Thr^{138} 'maintains' –rather than 'promotes'– the primed slowly releasable pool and, as a consequence, it also retains the release-ready vesicle pool (Nagy *et al.*, 2004). However, unlike PKC phosphorylation, PKA phosphorylation of SNAP-25 plays a less significant role in SNARE complex formation. Initial studies showed that PKA-dependent phosphorylation of SNAP-25 does not regulate ternary SNARE complex assembly and Thr^{138} is not essential for the complex formation or stability (Risinger and Bennett, 1999). Quite the opposite, a study *in vitro* reported that PKA activity on SNAP-25 at Thr^{138} inhibits the formation of SNARE complexes, decreasing the intensity of Western blot bands higher than the molecular weight of the native SNARE proteins (Gao *et al.*, 2016).

In conclusion, SNAP-25 phosphorylation by PKA and PKC contribute in different ways to the formation of the SNARE complex and provide remarkable data to understand the regulation of exocytosis.

SNAP-25 at the neuromuscular junction

SNAP-25 is a neural protein and is exclusively found in presynaptic terminals. dSTORM imaging at the mouse NMJ revealed a punctate distribution of SNAP-25 coincident with active zone proteins (Jones *et al.*, 2017) (■ Figure 10). We corroborated this observation with a confocal microscopy analysis. In adult rat diaphragm and LAL muscles, SNAP-25 is concentrated at the end of nerve terminals in areas close to active zones, without colocalization with Schwann cells (Simó *et al.*, 2019). The levels of both SNAP-25 and pSNAP-25 (Ser^{187}) in the diaphragm muscle were much more lower than in the CNS, remarkably high in the rat brain cortex (Simó *et al.*, 2019).

Subcellularly, total and phospho-Ser^{187} SNAP-25 are predominantly located in the membrane fraction (Simó *et al.*, 2019). The complete neuromuscular activity –presynaptic activity and muscle contraction– does not appear to affect SNAP-25 (Simó *et al.*, 2019). However, our results showed that synaptic activity alone enhances SNAP-25 Ser^{187} and that muscle contraction *per se* retrogradely inhibits this mechanism. In addition, the enhancement of SNAP-25 by presynaptic stimulation is located at the membrane fraction, suggesting an enhancement of the pool of primed vesicles and, by extension, over neurotransmission (Simó *et al.*, 2019).

Regarding the kinases responsible for such regulation, the PKCε isoform is essential to enhance both SNAP-25 phosphorylation and protein levels at the NMJ. This was studied using the $\varepsilon V_{1\text{-}2}$ inhibitor and phorbol esters. On the other hand, we did not find cPKCβI activity and TrkB signaling involved in the activity regulation of SNAP-25 (Simó *et al.*, 2019). This prompted us to explore the input of other signaling pathways like the purinergic and muscarinic, the latter studied in this thesis.

5.2. SM proteins: role of Munc18-1

SM (Sec1/Munc18-like) proteins are, together with SNARE proteins, the two universally required components of the intracellular membrane fusion machinery (Südhof and Rothman, 2009). They have been associated with

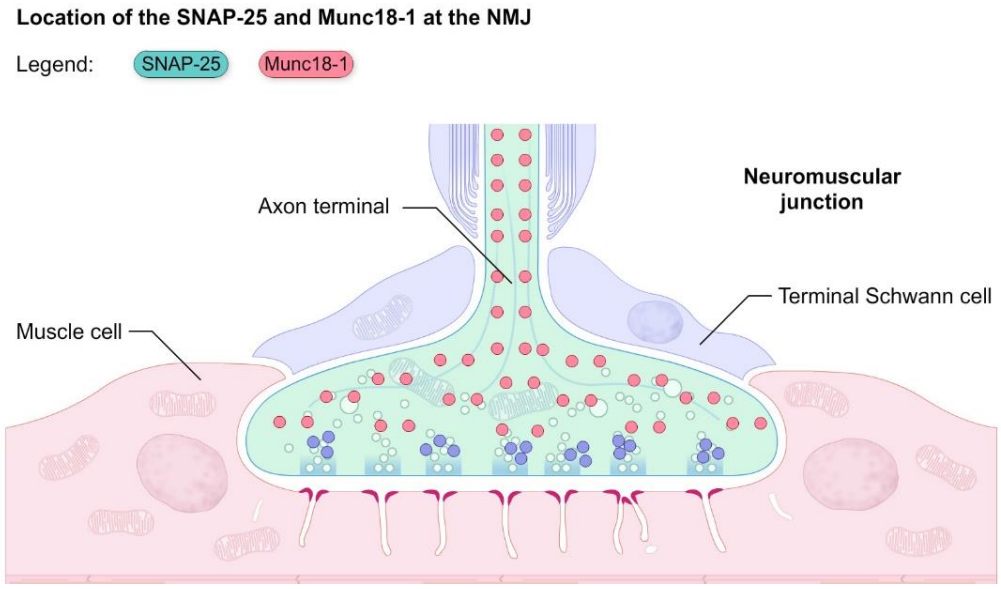

Figure 10. **Location of SNAP-25 and Munc18-1 at the NMJ.** SNAP-25 (turquoise) is exclusively found in punctate regions at the end of nerve terminals, coincident with active zones. The SM protein Munc18-1 is also found exclusively in motoneurons, present in nerve terminals and along axons. *Source: own elaboration.*

membrane fusion since the isolation of Mammalian homologue of Uncoordinated-18 (**Munc18**) bound to the t-SNARE syntaxin-1 (Hata *et al.*, 1993). The family of SM proteins has very specific vesicle/target functions. The major member of the SM proteins in synapses is **Munc18-1** (Südhof and Rothman, 2009).

SM proteins are composed of a conserved ~600 amino acid sequence with repetitive domains that folds into an arch or a 'hair claw'-shaped structure (■ Figure 9-D and 9-E). The fundamental function of SM proteins is to clasp a four-helix bundle. This structure can be found in either the four-helix bundle of a zippering SNARE core, or in Syntaxin-1 alone under a 'closed' conformation (Südhof and Rothman, 2009).

In the first stage of neurotransmission, Munc18-1 binds to an individual Syntaxin-1 from the membrane, folding together the four helices of Syntaxin-1 (Dulubova *et al.*, 1999). One of these helix domains contains the SNARE motif, so Munc18-1 hides and disables the spontaneous assemble of the core complex (Südhof, 1995; Wu *et al.*, 1998). The Syntaxin-1 conformation with all its four helices clasped together by the SM protein is known as the

'closed' conformation (Dulubova *et al.*, 1999). The early discovery of this mode of binding to the closed conformation of Syntaxin-1, incompatible with SNARE complex assembly, led to the idea that SM proteins were negative regulators. However, **the genetic deletion Munc18-1 blocks exocytosis** (Verhage *et al.*, 2000) more completely than the already strong effect of deleting the SNARE synaptobrevin (Schoch *et al.*, 2001). Indeed, Munc18-1 knockout mice are embryonically lethal and individual neurons lacking Munc18-1 die (Verhage *et al.*, 2000).

The question was resolved when a second, distinct mechanism of interaction between SM and SNARE proteins was found (■ Figure 9-F), explaining how SM proteins could promote fusion. In that, the SM protein is anchored to a specific N-terminal peptide sequence of the syntaxin (Yamaguchi *et al.*, 2002). This binding allows Syntaxin-1 to form a SNARE core, whose four helices are then clasped together by the SM protein (Munc18-1) near the membrane (■ Figure 9-F). Once clasped around a SNARE pin, exactly how SM proteins cooperate with SNARE complexes for fusion is not yet known (Südhof and Rothman, 2009). A kinetic role has been proposed in which SM proteins assemble SNAREs into a productive topology at the interface of the two membranes (such as a ring-like arrangement), possibly by restricting the diffusion of SNAREs into the space between fusing membranes (Rizo *et al.*, 2006).

Munc18-1 is an essential **PKC substrate**, being phosphorylated at serines 306, 312 and 313 (Ser^{313}) (de Vries *et al.*, 2000; Craig *et al.*, 2003). It is still not clear how this PKC phosphorylation influences neurotransmission. Molecularly, the PKC phosphorylation of Munc18-1 Ser^{306} and Ser^{313} *in vitro* reduces the ability to bind Syntaxin-1 (Fujita *et al.*, 1996). This generally causes a potentiation of neurotransmission. However, the functional result depends on the system: when Munc18-1 is replaced by a PKC-insensitive version (S306A, S313A), it abolishes post-tetanic potentiation in cultured hippocampal synapses (Wierda *et al.*, 2007), strongly decreases it at the calyx of Held (Genç *et al.*, 2014) but does not affect particular synapses from hippocampal and cerebellar slices (Wang *et al.*, 2021). On the other hand, stimulation induces Munc18-1 dispersion from hippocampal synapses and its

reclustering requires PKC and calcium influx (and not Syntaxin-1 binding) (Cijsouw *et al.*, 2014). This enhances synaptic strength, as synapses that recruit more Munc18-1 after stimulation also have a larger releasable vesicle pool (Cijsouw *et al.*, 2014).

Overall, Munc18-1 and the other SM proteins are essential to cooperate and organize spatially and temporally the assembly of the SNARE complex, being an important PKC substrate to stimulate fusion (Südhof and Rothman, 2009).

Munc18-1 at the neuromuscular junction

In recent years, our research team has been studying the function of Munc18-1 at the adult NMJ. We found that Munc18-1 and pMunc18-1 (pSer^{313}) are present in basal conditions at the diaphragm, spinal cord and brain samples (Simó *et al.*, 2018). Munc18-1 is enriched in the particulate fraction, in correspondence with its association with membranes and synaptic vesicles (Simó *et al.*, 2018).

Regarding the expression in neuromuscular junctions, Munc18-1 immunoreactivity is exclusively found in motoneurons (■ Figure 10), from their axons to the final presynaptic terminals, and it is absent in Schwann cells and postsynaptic myocytes (Simó *et al.*, 2018).

Munc18-1 levels are greatly influenced by neuromuscular activity. On the one hand, presynaptic stimulus increases both Munc18-1 expression and phosphorylation at Ser^{313} (Simó *et al.*, 2018). In contrast, postsynaptic muscle contraction retrogradely inhibits total and phospho-Munc18-1. Several treatments can reproduce these changes. For example, the PKC activator PMA increases Munc18-1 levels and high calcium media enhances Munc18-1 phosphorylation and downregulates its levels (Simó *et al.*, 2018) .

5.3. Regulatory proteins of the release machinery

The release machinery has many accessory proteins that ensure the specificity of the fusion process, adapting the universal fusion machinery to the physiological context (Südhof and Rothman, 2009).

Synaptotagmin and **complexin** are two of the most prominent membrane fusion regulators, working together to regulate Ca^{2+}-triggered fusion (Giraudo *et al.*, 2006). **Complexin** (also known as synaphin) acts upstream of membrane fusion by clamping the SNARE core complex like a clasp that blocks the complete zippering of SNARE complexes (Ishizuka *et al.*, 1995; Maximov et al., 2009). On the other hand, **Synaptotagmin** is an integral protein from synaptic vesicles that contains two PKC-like C2 domains, allowing it to sense the calcium concentration in the media. Synaptotagmin competes with complexin for binding to assembled SNARE pins, releasing complexin in a Ca^{2+}-dependent manner (Maximov *et al.*, 2009). Therefore, SNARE pins are first locked by complexin right after being formed. When an action potential arrives in the nerve terminal, Ca^{2+} flows into it and binds synaptotagmin, which reverses the action of complexin (Südhof and Rothman, 2009). This results in the complete fusion of the two membranes and the release of the content of the synaptic vesicle into the synaptic cleft.

The fusion of synaptic vesicles to the plasma membrane requires a previous vesicular trafficking to bring vesicles and target membrane into apposition. The Myristoylated Alanine-Rich protein Kinase C Substrate (**MARCKS**) is required in the trafficking of vesicles along the neurites (Yang *et al.*, 2002) due to its activity rearranging the cytoskeleton and interacting with membranes (Vaughan *et al.*, 1998). MARCKS $Ser^{152/156}$ is a major, specific substrate of PKC that is phosphorylated during neurosecretion (Nairn and Aderem, 1992). MARCKS is also a calmodulin-binding protein and binding of calmodulin inhibits phosphorylation of the protein by PKC (Hartwig *et al.*, 1992). MARCKS is located at the points of insertion of actin filaments to the plasma membrane (Nairn and Aderem, 1992). MARCKS is released to the cytoplasm after PKC activation or calmodulin binding in macrophages (Nairn and Aderem, 1992). Thus, MARCKS represents a platform for PKC and calmodulin signaling pathways to control the interaction of membranes with the actin cytoskeleton.

Finally, another important regulatory protein is the ATPase **NSF** (N-ethylmaleimide Sensitive Factor) and its adaptor protein **SNAP** (soluble NSF attachment protein, which is a different protein from SNAP-25). Once

exocytosis has occurred, NSF and SNAP bind directly to the SNARE pin in the membrane and NSF uses 3 to 6 ATPs to disassemble the complex for each catalytic cycle (Söllner *et al.*, 1993a; Mayer *et al.*, 1996). Thus, NSF and SNAP contribute to the recycling of SNARE core components for the following release events.

MARCKS at the neuromuscular junction

As a major PKC target, MARCKS has been of interest to our laboratory to report the activity of the kinase. Our research showed that MARCKS is associated with motoneurons in the muscle tissue (Obis *et al.*, 2015a). In particular, anti phospho-MARCKS labelling disappears in denervated *Extensor digitorum longus* muscle (EDL) together with Syntaxin labelling (Obis *et al.*, 2015a).

In basal conditions, the presynaptic nPKCε isoforms phosphorylate this substrate, as pMARCKS levels can be decreased by applying the specific nPKCε inhibitor $\varepsilon V_{1\text{-}2}$ (Obis *et al.*, 2015a). Because PKC is subject to activity-dependent degradation (Parker *et al.*, 1995; Lee *et al.*, 1996; Lu *et al.*, 1998; Kang *et al.*, 2000), the detection of MARCKS phosphorylation is very convenient to report PKC activity. For example, phospho-MARCKS levels increase after a presynaptic stimulus treatment at the rat diaphragm, helping to identify PKC activity in that condition even though nPKCε becomes activity-dependent degraded (Obis *et al.*, 2015a). On the other hand, muscle contraction *per se* enhances MARCKS phosphorylation further over the presynaptic stimulus, an action involving the TrkB signaling cascade.

In the CNS, MARCKS protein expression has been related to behavioral impairments (McNamara *et al.*, 2003) and its PKC-mediated phosphorylation occurs during learning and long-term potentiation (Ramakers *et al.*, 1999). Thus, we expect MARCKS at the neuromuscular junction to be implicated in the synaptic plasticity triggered by muscarinic signaling. In this thesis we used MARCKS as a PKC activity indicator and studied the influence of the M_1 and M_2 muscarinic receptors as well as the kinases PKCβI, PKCε and PKA.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
MUSCARINIC RECEPTOR MODULATION OF PROTEIN KINASE A, PROTEIN KINASE C AND EXOCYTOTIC PROTEINS AT THE NEUROMUSCULAR JUNCTION
Víctor Cilleros Mañé

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

Justification

The neuromuscular junction uses muscarinic acetylcholine receptors to auto-regulate neurotransmission. In the adult rodent skeletal muscle there are two functionally active subtypes, M_1 and M_2. M_1 classically enhances PKC and acetylcholine release whereas M_2 inhibits PKA and the release. However, the latest electrophysiological findings started to show complex cross-talking between muscarinic pathways. Thus, further molecular data of muscarinic signaling is now necessary to comprehend what happens underneath the extensive functional literature.

Therefore, the present thesis has been structured to characterize, firstly, how M_1 and M_2 mAChRs regulate the PKA pathway and secondly, how M_1 and M_2 mAChRs regulate the presynaptic PKC pathway.

Hypothesis

Therefore, the hypothesis of this thesis is:

The muscarinic receptors M_1 and M_2 regulate specific protein kinase A subunits and protein kinase C isoforms to modulate the phosphorylation of exocytotic proteins at the skeletal muscle neuromuscular junction.

Objectives of Publication 1

Main objective

To analyze how muscarinic receptors modulate the PKA pathway over exocytotic proteins at the neuromuscular junction.

Specific objectives

1. To determine whether there is interplay between M_1 and M_2 muscarinic acetylcholine receptor (mAChRs) subtypes in the skeletal muscle.

2. To determine the specificity of antibodies against PKA catalytic subunits (Cα, Cβ) and regulatory subunits (RIα, RIβ, RIIα, RIIβ).

3. To determine whether the PKA catalytic (Cα, Cβ) and regulatory (RIβ, RIβ, RIIα, RIIβ) subunits are modulated by M_1 and M_2 mAChRs in the skeletal muscle. Moreover, to determine whether PKA catalytic subunits are regulated by synaptic activity-induced stimulation.

4. To determine whether M_1 and M_2 mAChRs modulate the subcellular translocation of PKA subunits in the skeletal muscle.

5. To determine whether M_1 and M_2 mAChRs modulate the protein levels and the subcellular translocation of the scaffold AKAP150.

6. To determine the PKA subunits that are enriched in the synaptic region of the diaphragm. Moreover, to analyze whether M_2 mAChR affects the interaction between the RIβ subunit and the Cα and Cβ.

7. To determine whether M_1 and M_2 mAChRs modulate the phosphorylation of the PKA substrates CREB and SNAP-25 and the involvement of PKA.

Objectives of Publication 2

Main objective

To analyze how muscarinic receptors modulate the PKC pathway over exocytotic proteins at the neuromuscular junction.

Specific objectives

1. To determine whether the M_1 and M_2 muscarinic acetylcholine receptors subtypes modulate the protein levels and phosphorylation of the presynaptic PKCβI and PKCε isoforms in the skeletal muscle. Moreover, to determine the effect over the widely expressed PKCα.
2. To determine whether M_1 and M_2 mAChRs modulate the subcellular translocation of PKCβI and PKCε and their phosphorylated forms at the NMJ.
3. To determine whether PDK1 phosphorylation is induced by M_1 and M_2 mAChRs and whether PDK1 activity is linked to PKCβI and PKCε maturation and the phosphorylation of their substrates at the NMJ.
4. To determine whether M_1 and M_2 mAChRs modulate the protein levels and phosphorylation of the PKC substrates Munc18-1, SNAP-25 and MARCKS.
5. To determine the role of PKCβI, PKCε and PKA in each modulation of Munc18-1, SNAP-25 and MARCKS by M_1 and M_2 muscarinic receptors subtypes.
6. To determine whether M_1 and M_2 mAChRs modulate the translocation of Munc18-1, SNAP-25 and MARCKS at the NMJ.
7. To determine whether the signaling of M_2 mAChR over PKCβI, PKCε, Munc18-1 and SNAP-25 protein level and phosphorylation occurs at the synaptic region of the rat diaphragm.
8. To corroborate the location of NMJ nerve terminal of the PKCε, PKCβI, PDK1, Munc18-1 and SNAP-25.

CHAPTER 3

MATERIALS AND METHODS

UNIVERSITAT ROVIRA I VIRGILI
MUSCARINIC RECEPTOR MODULATION OF PROTEIN KINASE A, PROTEIN KINASE C AND EXOCYTOTIC PROTEINS AT THE NEUROMUSCULAR JUNCTION
Víctor Cilleros Mañé

CHAPTER 3

MATERIALS AND METHODS

1. Animal care and tissue isolation

1.1. Animal care

The animals were cared for following the European Community Council Directive guidelines for the humane treatment of laboratory animals. Animal handling and treatments were approved by the *Comitè Ètic d'Experimentació Animal* from *Facultat de Medicina i Ciències de la Salut (Universitat Rovira i Virgili)* in accordance with the *Llei 5/1995* and *Decret 214/1997 de la Generalitat de Catalunya* and the *Real Decreto Español 53/2013* (published in BOE 34, 08/02/13) which establishes the canons of animal protection to follow in experimentation and other scientific purposes.

This thesis used adult male and female *Sprague-Dawley* rats (Criffa, Barcelona, Spain). The animals were maintained on the animal facility of the *Facultat de Medicina i Ciències de la Salut (Universitat Rovira i Virgili)* in a standard cage of Makrolon (23 x 46 x 14 cm^3) and were housed at constant temperature (22 ± 2°C), relative humidity (50 ± 10%) and automatic light cycles (12h light/dark). Food (Panlab rodent chow, Barcelona, Spain) and tap water were offered *ad libitum* throughout the study.

1.2. Tissue dissection

Adult male and female *Sprague-Dawley* rats (40-80 days; Criffa, Barcelona, Spain) were euthanized for tissue harvest and analysis. Unconsciousness was induced by an intraperitoneal injection of tribromoethanol 2% (0.15 ml/10 g body weight) and afterwards euthanasia was performed through anesthetic overdose. As indicated in the Directive 2010/63/EU and the *Real Decreto* 53/2013, death was confirmed by exsanguination. This method prevents the formation of blood clots during the dissection. The muscle tissues, diaphragm and LAL, were harvested immediately after euthanasia.

The diaphragm as a neuromuscular junction model

The **diaphragm** is a classic model to study the neuromuscular junction. It is composed of mixed muscle fibers (55% slow twitch fibers; 25% fast twitch oxidative-glycolytic fibers; and 20% fast twitch glycolytic fibers (Rochester, 1985). The contraction of these fibers is controlled by the left and right **phrenic nerves** (■Figure 11-A), which supply each hemidiaphragm from the C3, C4 and C5 ventral roots in rodents (Meyerholz *et al.*, 2018). The phrenic nerve also contains many sensory nerve fibers that supply the fibrous pericardium, the mediastinal pleura, and central areas of the diaphragmatic pleura. Sympathetic postganglionic nerve fibers also travel with this nerve. The coordination of the diaphragm relies on the central control of lower motoneuron firing through dendrite bundles in the spinal cord. The diaphragm consists of a muscular component and a central tendon (■ Figure 11-B). The muscle can be subdivided in the costal and the crural (crus) diaphragms. In rodents, the costal region has well distinguished left and right parts called **hemidiaphragms** (Stuelsatz *et al.*, 2012). In the experiments of this thesis, the diaphragm was excised together with the phrenic nerves. We took special care to isolate the same nerve length (maximum length of ~1 cm from the muscle contact). At the end of the dissection, nerve connectivity was checked by pinching the tip of each nerve and observing a muscle twitch.

The Levator auris longus (LAL) as a neuromuscular junction model

The ***Levator auris longus*** (LAL) muscle was described by Angaut-Petit *et al.*, 1987. It is located at the back of the neck, underneath the skin. The LAL consists in two muscles, left and right, which emerge from the midline and extend towards the base of the auricles (■ Figure 11-C). Each of these muscles is subdivided into two fascia, cranial and caudal, which respectively insert to the anterior and posterior parts of the pinna. The LAL myocytes are mainly fast-twitch muscle fibers (Eržen *et al.*, 2000) and are controlled by the posterior auricular branch of the facial nerve (■ Figure 11-D). Motoneurons enter into the LAL from its lateral edges (Angaut-Petit *et al.*, 1987). The LAL is very thin, constituted by just five to six layers of muscle cells. This flat shape and thinness facilitates the observation of motor endplates without tissue sections (Tomas et al., 2000; Lanuza et al., 2001; Burke et al., 2018).

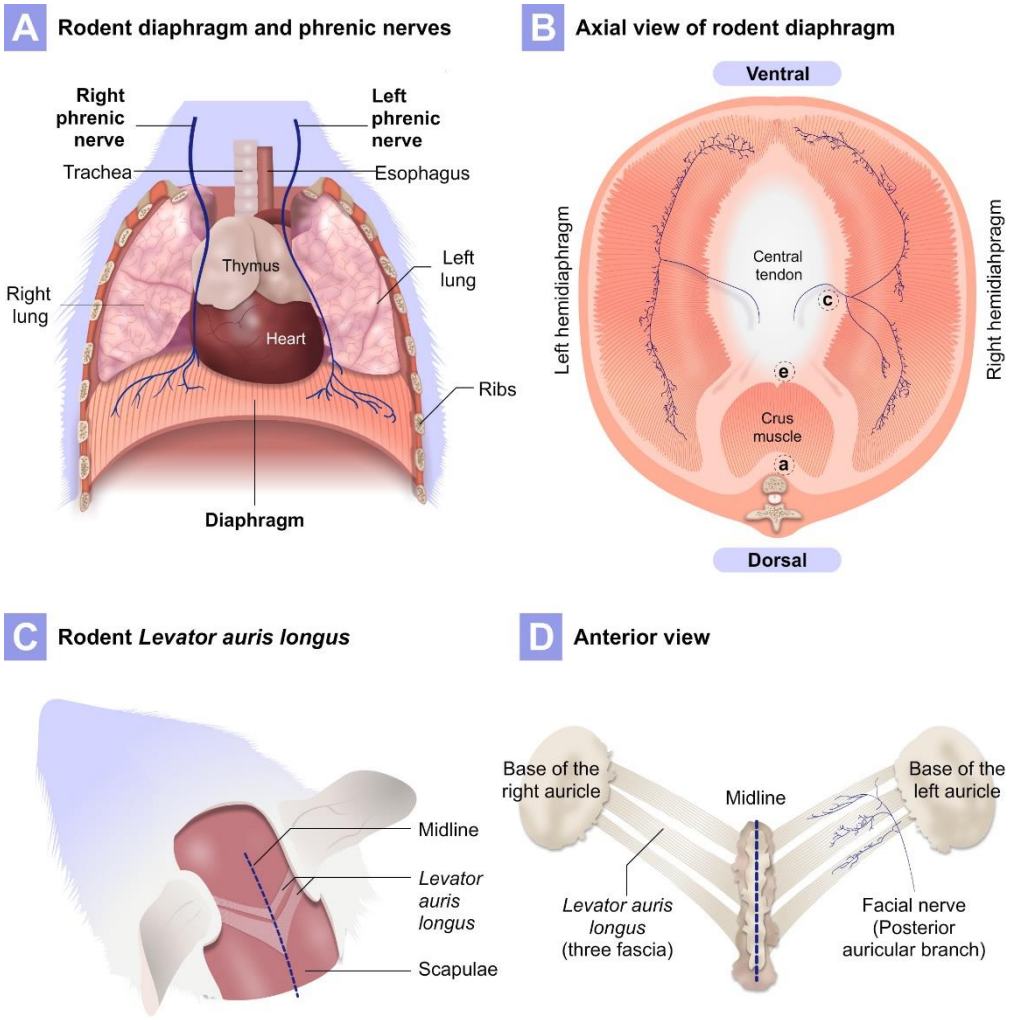

Figure 11. **Anatomy of the rodent's diaphragm and *Levator auris longus*.** A Thoracic wall, lungs and mediastinum. The left and right phrenic nerves descend inside the mediastinum and supply both sides of the diaphragm. In rats, the right phrenic nerve travels more in contact with the pericardium, has pericardial branches and thus has to be isolated more carefully from the surrounding tissues. In contrast, the rat left phrenic nerve is only attached to a loose layer of thin of connective tissue and can be more easily dissected. B The rat diaphragm consists of two left and right hemidiaphragms, joined by the central tendon. The phrenic nerves makes contact with the diaphragm at the central region, where they branch out throughout the muscle's length. In our dissection procedure, we eliminate asymmetric regions like the crus muscle, the openings of the vena cava (c), the esophagus (e) and the aorta (a). C The rat *Levator auris longus* (LAL) is located just underneath the skin and is the most superficial muscle at the back of the neck. It emerges from the midline and extends towards the base of the auricles. D The rat LAL consists of two left and right muscles separated by the midline. The motoneurons supplying the LAL derive from the posterior auricular branch of the facial nerve. They enter the muscle from its lateral edge towards the medial part. *Source: own elaboration based on* (Stuelsatz *et al.*, 2012; Burke *et al.*, 2018; Meyerholz *et al.*, 2018).

2. Treatments and sample processing

2.1. Chemicals

Isolated nerve-muscle diaphragm preparations were immersed in **Ringer's solution** (mM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 12, glucose 12.1). This solution was oxygenated with carbogen 95% oxygen and 5% carbon dioxide (95:5 O_2:CO_2) and maintained at 26°C.

All chemicals (Table 6) were applied diluted in Ringer's solution and both control and drug-containing solutions contained dimethyl sulfoxide (DMSO) at final concentration 0.1% (v/v). DMSO dissolves polar and non-polar compounds and is commonly used as a vehicle both *in vivo* and *in vitro*.

Muscarinic inhibitors

Pirenzepine dihydrochloride (Pir), from Tocris, was stored in a 10 mM stock in milli-Q water and used at 10 µM in Ringer's solution (Santafé *et al.*, 2007). Pir is a muscarinic receptor antagonist with high affinity for M_1 muscarinic receptors. It displays a 57-fold greater affinity for the M_1 than for the M_2 subtype in *in vitro* assays (Hammer *et al.*, 1980; Buckley *et al.*, 1989).

Methoctramine tetrahydrochloride (Met), from Sigma, was stored in a 1 mM stock in milli-Q water and used at 1 µM in Ringer's solution (Santafé *et al.*, 2007). Met is a muscarinic receptor blocker with high affinity for M_2 muscarinic receptors. In *in vitro* assays, Met shows a 4.4-fold greater affinity for the M_2 receptor subtype than for the M_1 subtype (Giraldo *et al.*, 1988; Buckley *et al.*, 1989).

Atropine (Atr), from Sigma, was stored in 200 µM stock in milli-Q water and used at 2 µM in Ringer's solution (Santafé *et al.*, 2007). Atr is a well-referenced pan-muscarinic blocker used in clinical practice (Birdsall *et al.*, 1978; Burford and Nahorski, 1996; Slutsky *et al.*, 1999; Kociolek *et al.*, 2006). This drug has been extensively used to study muscarinic activity in previous electrophysiological studies (Santafé *et al.*, 2006, 2007; Obis *et al.*, 2015b). The affinity of Atr for M_1 is 9.0–9.7 (log affinity constant or pK_B value) and for M_2 9.0–9.3, which is practically the same for both mammalian subtypes (Caulfield and Birdsall, 1998).

Table 6. Chemicals and treatment information.

Target	Chemical	Abbreviation	Working solution	Source
M_1 mAChR	Pirenzepine	Pir	10 µM	Tocris
M_2 mAChR	Methoctramine	Met	1 µM	Sigma
M_1 and M_2	Atropine	Atr	2 µM	Sigma
PKA	H-89	H-89	5 µM	Calbiochem
PKCβI	PKCβI inhibitor peptide	βIV_{5-3}	10 µM	Provided by Dr. Mochly-Rosen
PKCε	PKCε inhibitor peptide	εV_{1-2}	100 µM	MERCK
PDK1	GSK2334470	GSK-470	2 µM	Sigma
VGSC	µ-conotoxin GIIIB	µ-CTX	1.5 µM	Alomone

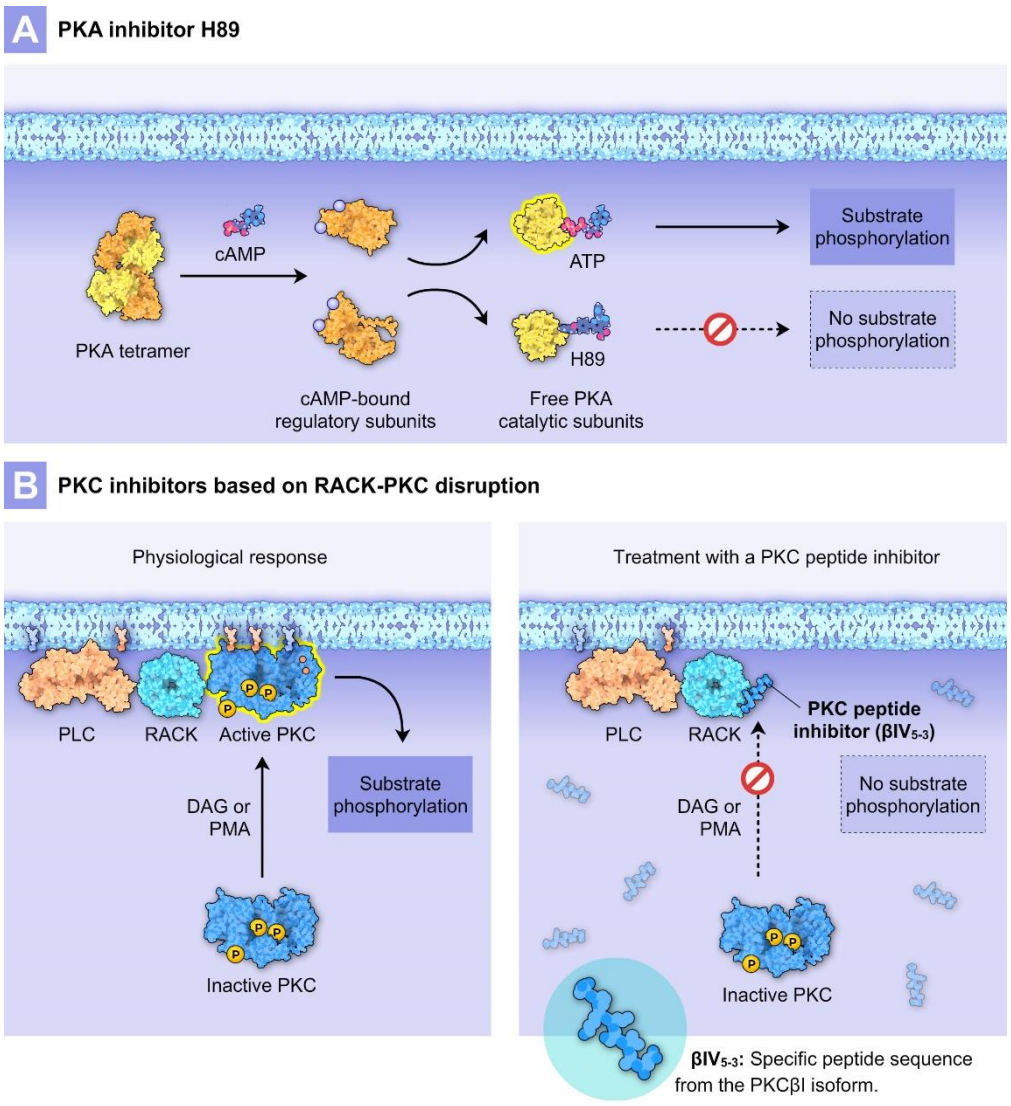

Figure 12. **PKA and PKC blockers used in the study.** A H-89 is a PKA inhibitor which competes to bind the ATP-binding site from catalytic subunits. This blocks the source of phosphoryl groups and, thus, kinase activity. B The PKC inhibitors compete with the isoforms to bind RACK1 protein. This blocks their targeting to the correct subcellular location and therefore their activation. *Source: own elaboration.*

PKA inhibitors

PKA activity was blocked with N-[2-((p-Bromocinnamyl)amino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (**H-89**), from Calbiochem. H-89 was stored in a 5 mM stock in milli-Q water and used at 5 µM (Santafé *et al.*, 2007). This drug blocks kinase activity through competitive inhibition for the ATP-binding site of PKA catalytic subunits (■ Figure 12-A).

PKC inhibitors

PKC activity was blocked with PKC-derived peptides which compete for RACK1 binding and disrupt the cellular targeting of PKC isoforms (■ Figure 12-B). Because each peptide derives from a particular PKC isoform, it only competes with that particular RACK-PKC isoform interaction. The PKCβI was blocked with the **PKCβI inhibitor βIV$_{5-3}$ peptide** (βIV$_{5-3}$) (Liu *et al.*, 1999; Zhang *et al.*, 2015), from Dr. Mochly-Rosen from Stanford University, and PKCε was blocked with the **PKCε inhibitor εV$_{1-2}$ peptide** (εV$_{1-2}$) (Johnson *et al.*, 1996) from MERCK. Both peptides are <40 amino acids (βIV$_{5-3}$, CKLFIMN; εV$_{1-2}$, EAVSLKPT). Briefly, studies show that blocking PKCβI with βIV$_{5-3}$ did not affect PKCβII (Stebbins and Mochly-Rosen, 2001) and blocking PKCε with εV$_{1-2}$ did not affect the novel PKCδ or classical PKC isoforms (Johnson *et al.*, 1996; Mochly-Rosen and Gordon, 1998; Way *et al.*, 2000). Furthermore, εV$_{1-2}$ peptide has been validated with PKCε knockout mice (Khasar *et al.*, 1999; Di-Capua *et al.*, 2003). Also, multiple sequence alignment by ClustalW reveals that βIV$_{5-3}$ peptide shares 100% identity with PKCβI (Uniprot ID: P68403-1) and 0% identity with PKCε (Uniprot ID: P09216) or PKCβII (Uniprot ID: P68403-2). Additionally, εV$_{1-2}$ peptide shares 0% identity with PKCβI and 100% identity with PKCε. Working concentration was optimized to 10 µM for βIV$_{5-3}$ (Hurtado *et al.*, 2017a) and 100 µM for εV$_{1-2}$ (Obis *et al.*, 2015a). The difference in concentration is due to βIV$_{5-3}$ peptide being connected to a deliverer peptide to enhance cell penetration.

PDK1 inhibition

PDK1 activity was blocked with GSK2334470, also known as GSK-470 (#SML0217 Sigma MERCK). This inhibitor is highly specific: it has an IC50 of ~10 nM for PDK1, but does not suppress the activity of 93 other protein kinases, including PKC and PKA, at 500-fold higher concentrations (Najafov *et al.*, 2011). Higher concentrations have been tested in cell lines and mice (Najafov *et al.*, 2011; Yang *et al.*, 2017; Zhang *et al.*, 2018). As for treatment duration, the maximal inhibition of GSK2334470 in HEK293 cultures is observed within 10 min and sustained for at least 2 h, longest point examined. Based on the literature, GSK2334470 was made as 5 mM and we applied a concentration of 2 μM for 30 minutes on excised diaphragm muscles.

Contraction inhibition

In Chapter 1 – Objective 3 we studied the muscarinic signaling and PKA expression under phrenic nerve stimulation. Our aim was to understand if the phrenic nerve stimulation does *per se* affect the protein levels for Cα and Cβ. Therefore, postsynaptic contraction was blocked to allow the comparison of our results with the previous electrophysiological data and, furthermore, to avoid the multiple retrograde changes that muscle contraction induces over the signaling and protein expression in nerve terminals (Hurtado *et al.*, 2017a; Simó *et al.*, 2018).

Muscle contraction was blocked using μ-conotoxin GIIIB (#C-270, Alomone Labs Ltd, Jerusalem, Israel). This toxin selectively inhibits sarcolemmal voltage-gated sodium channels (VGSCs) without affecting the synaptic ACh release and has been used extensively for electrophysiology studies at the diaphragm (Favreau *et al.*, 1999; Santafé *et al.*, 2001, 2009; Mantilla *et al.*, 2014). μ-conotoxin GIIIB was acquired as lyophilized powder of >99% purity and the working solution was 1.5 μM.

2.2. Treatments

We used selective and non-selective blockers to study the muscarinic signaling pathway at the diaphragm neuromuscular junction. These treatments were applied to freshly excised diaphragm preparations under oxygenated Ringer's solution. Thanks to the following pairwise experimental design, we were able to control interindividual variation. Each diaphragm was divided into two hemidiaphragms, right and left (■ Figure 13-A). One of them underwent the treatment while the other served as a paired control. The assignment of the treatment to the right or left hemidiaphragm was random and annotated to ensure homogeneity between groups. No left–right difference was observed during this work.

In **single-inhibitor treatments**, the treated preparation was incubated in Ringer's solution containing the appropriate inhibitor, whereas the control preparation was incubated without it (sham incubation) (■ Figure 13-B). The first 30 minutes after the drug was added to the media were considered preincubation time. After that, the treatment was applied for 30 minutes. Both treated and control experiments lasted a total of 60 minutes. This period allows (1) cell penetration of the chemicals, (2) homogeneity of the time variable between stimulated and non-stimulated experimental setups and (3) the comparison with previous studies in the same model including electrophysiological studies (Santafé *et al.*, 2003; Obis *et al.*, 2015b; Hurtado *et al.*, 2017a).

Double-inhibitor treatments were performed to study if a muscarinic signaling response required a specific kinase (PKA, cPKCβI or nPKCε). In this kind of experiments, the treated hemidiaphragm was first preincubated for 30 minutes in Ringer's solution containing a kinase blocker –Drug 1– and afterwards for further 30 minutes in Ringer's solution containing the kinase blocker plus the muscarinic inhibitor specified –Drug 1 and 2– (■ Figure 13-C). The corresponding paired control was incubated for 60 minutes in Ringer's solution containing just the kinase blocker to discard the effects of the kinase inhibitor. Therefore, this experimental setup allows the observation of the muscarinic signaling without the activity of a particular kinase.

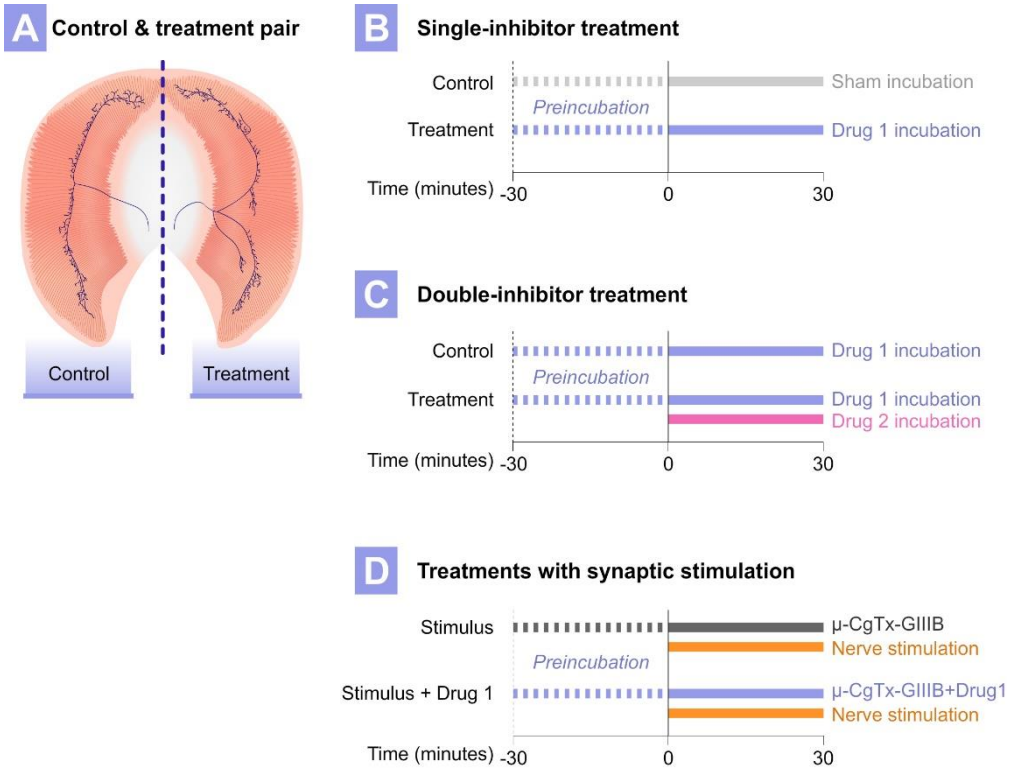

Figure 13. **Treatment setup.** A The experimental design was pairwise: control and treated preparations derive from the same animal. One hemidiaphragm is treated whereas the other serves as control. Left and right muscles were assigned randomly to the treatment in each experiment replicate. B In the single-inhibitor treatments the drug (Drug 1, in blue) was preincubated for 30 minutes and then the treatment applied for 30 minutes more. The control sham incubation contained Ringer's solution without drug. C The double-inhibitor treatments were used to determine if a previous incubation with one drug (Drug 1, in blue) modified the effect of a second drug (Drug 2, in pink). The control contains Drug 1 for normalization. D Treatments with presynaptic stimulation were similar to (B): the drug (Drug 1, in blue) was preincubated together with μ-conotoxin GIIIB for 30 minutes and then stimulated for 30 minutes more (Nerve stimulation, in orange). The pair control was incubated with μ-conotoxin GIIIB and stimulated for normalization. *Source: own elaboration.*

Thirdly, we also studied muscarinic blockers under the effect of **presynaptic stimulation of the phrenic nerve** (■ Figure 13-D). In this kind of experiments, the treated preparation is preincubated for 30 minutes in Ringer's solution containing a specific blocker –Drug 1– and μ-conotoxin GIIIB, a muscle contraction inhibitor. Afterwards, the phrenic nerve of the hemidiaphragm is stimulated for 30 minutes. The corresponding pair control is preincubated with μ-conotoxin GIIIB and stimulated without the blocker to study to discard the effect of presynaptic stimulation *per se*.

2.3. Tissue homogenization and fractionation

Whole cell lysates

After being treated, the muscles were immediately frozen in liquid nitrogen. Homogenization was performed with an overhead stirrer (VWR International, Clarksburg, MD) in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl 50 (pH 7.4), EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail 1% (Sigma, Saint Louis, MO, USA)). Insoluble materials were removed with two centrifugations at 4ºC: 1000g for 10 minutes and 15000g for 20 minutes. The final supernatant contained the whole cell fraction lysate.

Membrane/cytosol fractionated lysates

Unlike whole cell lysates, membrane-cytosol fractionation requires non-frozen samples without detergent-free lysis buffer. This is because both steps (freezing samples and detergent lysis) damage lipid membranes and prevent the correct fractionation. Thus, these samples were immediately homogenized after treatment (without a freezing step) and with a detergent-free lysis buffer (in mM: NaCl 150, Tris-HCl 50 (pH 7.4), EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; and protease inhibitor cocktail 1%). Insoluble materials were removed by centrifugation at 1000g for 15 minutes at 4ºC. The resulting supernatant was further centrifuged at 130000g for 1 hour. The new supernatant corresponded to the cytosolic fraction while the pellet to the membrane fraction. The latter was resuspended in detergent-containing lysis buffer (same composition as the whole cell lysis buffer described before). The purity of the subcellular fractionation was validated by Western blotting of the fraction-specific proteins GAPDH for cytosol and Na+/K+-ATPase for membrane.

Synaptic and extrasynaptic lysates.

Some experiments required to study the synaptic and extrasynaptic areas of the diaphragm. The phrenic nerve usually innervates the center of the myotubes, creating in the medial line across the hemidiaphragm a region

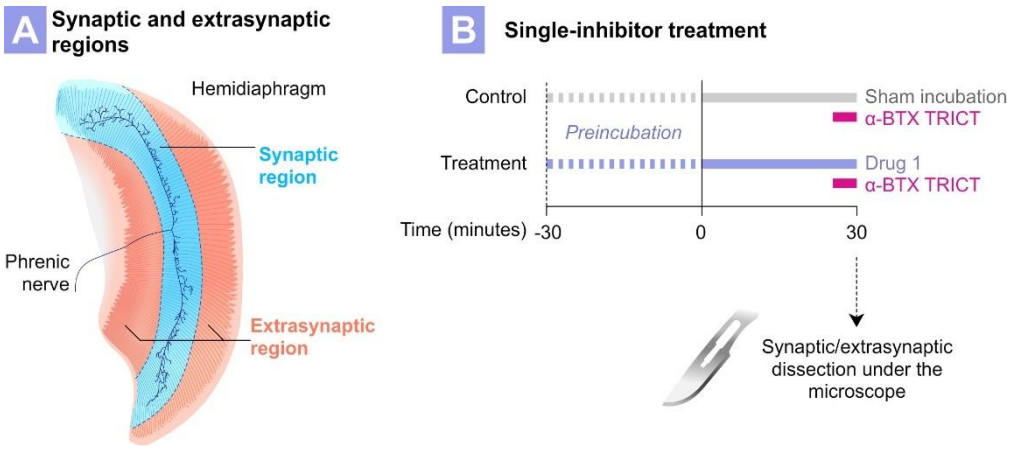

Figure 14. **Synaptic and extrasynaptic regions of a rat hemidiaphragm.** A The phrenic nerve innervation is concentrated in the medial line of the hemidiaphragm, making it possible to separate the NMJ-enriched synaptic region (light blue) from the extrasynaptic regions (orange). B Experimental setup for the synaptic and extrasynaptic experiments. One hemidiaphragm is treated whereas the other serves as control. The last 5 minutes of treatment, α-BTX conjugated with TRICT is added to the media, which marks NMJs and allows the dissection under the microscope. NMJ, neuromuscular junction; α-BTX, α bungarotoxin. *Source: own elaboration.*

rich in NMJs–the **synaptic region**–and two side regions where NMJ are very rare –the **extrasynaptic region**– (■ Figure 14-A).

This separation was performed as indicated in Besalduch *et al.*, 2010. First, the experiment is carried out as usual: one hemidiaphragm undergoes the treatment whereas the other serves as control (■ Figure 14-B). The preincubation and treatment are applied for 30 minutes and, in the last 10 minutes, 1/800 α-bungarotoxin (α-BTX) conjugated to tetramethyl-rhodamine (TRICT) (Molecular Probes) is added to the media. This short time allows to slightly mark NMJs and minimize the time α-BTX is present. Afterwards, the tissue is observed under a fluorescent light microscope and dissected. The separation is carried out at the end of the treatment to avoid the effects of slicing the tissue. Once separated, synaptic and extrasynaptic regions are immediately frozen with liquid nitrogen and lysed as whole cell samples (see the previous *Whole cell lysates* section for the buffer composition and procedure). We performed control experiments to check that our separation protocol was accurate by incubating some diaphragms with α-BTX and staining the nerves with an anti-neurofilament antibody. We did not detect any nerve or postsynaptic area in the extrasynaptic region.

2.4. Other samples – Knockout cell lines

We validated the anti-PKA antibodies against knockout lysates to guarantee the discrimination between isoforms. The knockout lysates were acquired from Novus Bio (Bio-techne). Each knockout lysate came with a control vial of the parental non-knocked cell lysate. The lyophilized lysates were resuspended in 100 µL sample buffer (2% SDS, 60 mM Tris-HCl pH 6.8, 10% glycerol, 60 mM β-mercaptoethanol and ~0.02% bromophenol blue). The volume was aliquoted and stored at –80°C to avoid freeze-thawing cycles. The details of these samples are summarized in Table 7.

Table 7. PKA knockout cell lines.

Sample (Ref.)	Gene	Cell line	Preparation method
Cα knockout (NBP2-65840)	PRKACA	293T	Knockout achieved by using CRISPR/Cas9. 2 bp insertion in exon 4.
Cβ knockout (NBP2-64806)	PRKACB	HeLa	Knockout achieved by using CRISPR/Cas9. 2 bp deletion in exon 1 and 1 bp insertion in exon 1.
RIα knockout (NBP2-65698)	PRKARIA	293T	Knockout achieved by using CRISPR/Cas9. 22 bp deletion in exon 1; 5 bp deletion in exon 1 and 1 bp insertion in exon 1.
RIIα knockout (NBP2-65316)	PRKAR2A	HeLa	Knockout achieved by using CRISPR/Cas9. 4 bp deletion in exon 1 and 1 bp deletion in exon 1.

3. Western blotting

3.1. General procedure

First, the protein content of the samples was determined by the DC protein assay (Bio-Rad, CA, USA). Volumes containing 30 µg of protein were loaded in an 8% SDS-PAGE gel (10% to detect SNAP-25), separated for 10 min at 90V and 60 min at 110V, and electrotransferred to PVDF membranes (Bio-Rad, CA, USA).

Next, membranes were stained with Sypro Ruby protein blot stain (Bio-Rad, CA, USA) to assess the **total protein** transference (Aldridge *et al.*,

2008). This stain provides a **loading control** highly reflective of quantitative differences in protein concentration. Additionally, it is compatible with the following blocking steps. Recent evidence demonstrate that total protein staining is more linear and reliable than high-abundance loading controls like β-actin (Aldridge *et al.*, 2008; Welinder and Ekblad, 2011; Gilda and Gomes, 2013; Steinberger *et al.*, 2015). In particular, common housekeeping proteins are sometimes differentially expressed in some conditions due to their activity- or age-dependent expression (Moskowitz and Oblinger, 1995; Dittmer and Dittmer, 2006; Liu and Xu, 2006; Montero-Melendez and Perretti, 2014; Chen and Xu, 2015).

Blocking solutions were TBST containing 5% of either nonfat dry milk, BSA (Sigma) or phosphoBlocker (AKR-103, Cell Biolabs). The primary antibodies were diluted in blocking solution and incubated overnight at 4°C. The HRP-conjugated secondary antibodies were incubated for 1 hour. Chemiluminescence was revealed with an ECL kit (GE Healthcare Life Sciences, UK) and imaged with the ChemiDoc XRS+ Imaging System (Bio-Rad, CA, USA). We used ImageJ to calculate the optical density of the bands, always from the same blot image. Differences were normalized against (1) background values and (2) the total protein transferred on the PVDF membranes, analyzed with Sypro Ruby protein blot stain (Aldridge *et al.*, 2008).

The ratios between the experiment and control were calculated from the same membrane image. All presented data derive from densitometry measurements made of 3-10 separate replicates, plotted against controls. For Western blot design no blinding was performed.

3.2. Antibodies and specificity tests

Table 8 lists the antibodies used in this thesis and their specifications. These antibodies presented bands of the predicted molecular weight without near prominent unspecific bands. The anti-M_1 and anti-M_2 antibodies were tested by subcellular fractionation and antigen preincubation. The corresponding bands of these GPCRs were enriched in the membrane fraction, absent in the cytosolic fraction and antigen preincubation strongly reduced them. We determined the specificity of the anti-SNAP-25 antibody in

Simó *et al.*, 2019. In this thesis we tested the specificity of anti-PKA subtypes antibodies with knockout cell lines (Results, Chapter 1); the PKA responsiveness of anti-pSNAP-25 Thr^{138} antibody with H-89 (Chapter 1); and the PDK1 responsiveness of anti-pPDK1 with GSK234470 (Chapter 2).

4. Immunohistochemistry

Several proteins were localized at the NMJ of diaphragm and LAL muscles by immunohistochemistry with identical results. The thinness of LAL muscles improved the imaging and analysis of NMJs. Whole muscle mounts were fixed with 4% paraformaldehyde for 30 minutes. After fixation, the muscles were rinsed with PBS and incubated in 0.1 M glycine in PBS. Afterwards, PBS supplemented with 1% Triton X-100 and 4% BSA was used for permeabilization and blockade of nonspecific binding. Then, muscles were incubated overnight at 4°C in a mixture of primary antibodies raised in different species (anti-Cβ subunit and anti-S100 to label Schwann cells) and then rinsed. The muscles were then incubated for four hours at room temperature in a mixture of appropriate secondary antibodies. nAChRs were detected with α-BTX conjugated with TRITC. Finally, whole muscles were mounted in Mowiol medium (from Calbiochem), which required overnight curing before observation. At least three muscles were used as negative controls and no cross-reaction was detected between antibodies. In some muscles, plastic embedded semithin sections (0.5 µm) were obtained for high-resolution immunofluorescence analysis of the neuromuscular junction molecules as previously described (Lanuza *et al.*, 2007). Immunolabelled NMJs from the whole-mount muscles were viewed with a laser-scanning confocal microscope (Nikon TE2000-E). Special consideration was given to the possible contamination of one channel by another. In experiments involving negative controls, the photomultiplier tube gains and black levels were identical to those used for a labelled preparation made in parallel with the control preparations. Images were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA) and neither the contrast nor brightness were modified.

Table 8. Antibodies. List of antibodies used in this thesis and specifications. Abbreviations: Hu, human; IHC, immunohistochemistry; Dil, dilution; Dk, donkey; Rb, rabbit; Ms, mouse; mAb, monoclonal antibody; pAb, polyclonal antibody; WB, Western blot.

Target	Immunogen	Origin	Company (Ref.)	WB Dil.	IHC Dil.
AKAP150	Rat AKAP150 residues 428–449.	Rb pAb	Millipore (07-210)	1/1000	–
ATPase	Chicken ATPase residues 27–55	Ms mAb	DSHB (a6f)	1/2000	–
CREB	Hu CREB synthetic peptide.	Rb pAb	CST (9192)	1/1000	–
CREB (pSer^{133})	Hu CREB residues around pSer^{133}	Rb pAb	CST (9191S)	1/1000	–
GAPDH	Rb GAPDH	Ms mAb	Santa Cruz (sc-32233)	1/2000	–
M_1 mAChR	Hu M_1 mAChR residues 227–353	Ms mAb	Alomone (AMR-001)	1/2000	–
M_2 mAChR	Hu M_2 mAChR residues 168–192	Ms mAb	Abcam (ab90805)	1/2000	–
MARCKS	Hu MARCKS residues 2-66	Ms mAb	Santa Cruz (sc-100777)	1/1000	–
MARCKS (pSer$^{152/156}$)	Rat MARCKS residues around pSer$^{152/156}$	Rb pAb	Sigma (07-1238)	1/1000	–
Munc18-1	Hu Munc18-1 residues around Tyr^{157}	Rb mAb	CST (13414)	1/1000	1/5000
Munc18-1 (pSer^{313})	Hu Munc18-1 residues 307–319	Rb pAb	Abcam (ab138687)	1/1000	–
PDK1	Hu PDK1 residues 229–556	Ms mAb	Santa Cruz (sc-17765)	1/1000	1/1000
PDK1 (pSer^{241})	Hu PDK1 residues around pSer^{241}	Rb pAb	CST (3061)	1/1000	–
PKA Cα	Hu Cα C-terminus.	Rb pAb	Santa Cruz (sc-903)	1/1000	–
PKA Cβ	Hu Cβ C-terminus.	Rb pAb	Santa Cruz (sc-904)	1/1000	1/1000
PKA RIα	Hu RIα residues 1–381.	Ms mAb	Santa Cruz (sc-136231)	1/1000	–
PKA RIβ	Hu RIβ C-terminus.	Rb pAb	Santa Cruz (sc-907)	1/1000	–
PKA RIIα	Ms RIIα C-terminus.	Rb pAb	Santa Cruz (sc-909)	1/1000	–
PKA RIIβ	Hu RIIβ residues 21–110.	Ms mAb	Santa Cruz (sc-376778)	1/1000	–
PKCα	Hu PKCα C-terminus	Rb pAb	Santa Cruz (sc-209)	1/1000	–
PKCα (pSer^{657})	Hu pPKCα residues 654–663	Rb pAb	Upstate (06-822)	1/1000	–
PKCβI	Hu PKCβI C-terminus	Rb pAb	Santa Cruz (sc-209)	1/1000	1/1000
PKCβI (pThr^{642})	Hu pPKCβI residues 640–644	Rb pAb	Abcam (ab75657)	1/1000	–
PKCε	Hu PKCε C-terminus	Rb pAb	Santa Cruz (sc-214)	1/1000	1/1000
PKCε (pSer^{729})	Hu PKCε residues around pSer^{729}	Rb pAb	Santa Cruz (sc-12355)	1/1000	–
SNAP-25	Hu SNAP-25 residues around Gln^{116}	Rb mAb	CST (5309)	1/1000	–
SNAP-25 (pSer^{187})	Rat SNAP-25 residues around pSer^{187}	Rb pAb	Abcam (ab169871)	1/1000	–
SNAP-25 (pThr^{138})	Hu SNAP-25 residues around Thr^{138}	Rb pAb	Biorbyt (orb163730)	1/1000	–
S-100	Purified bovine S100 protein	Rb pAb	Dako (Z0311)	–	1/1000
Secondary antibodies	Anti-Rb conjugated HRP	Dk pAb	Jackson (711-035-152)	1/10000	–
	Anti-Ms conjugated HRP	Rb pAb	Sigma (A9044)	1/10000	–
	Anti-Rb conjugated Alexa Fluor 488	Dk pAb	Molecular Probes (A-31573)	–	1/300
	Anti-Ms conjugated Alexa Fluor 488	Dk pAb	Molecular Probes (A-21202)	–	1/300

5. Co-Immunoprecipitation

Co-Immunoprecipitation (co-IP) is used to study interactions between proteins. This technique isolates protein complexes from a lysate using an antibody specific against one of the proteins. To precipitate and obtain the complex, the antibody needs to be bound to a solid substrate or resin (■ Figure 15-A), allowing to separate the protein from the solution. The "co" in co-immunoprecipitation experiments refers to the mild conditions (pH, temperature, salt concentration) used in the experimental assay to preserve other proteins tightly bound to the antibody-specific protein. Therefore, the antibody will not only precipitate its specific protein, but will also "pull down" those proteins that are interacting with it. In co-IP terms, the protein specifically captured with the antibody is called bait protein (or simply **bait**) and any protein co-precipitated with it is called prey protein (or simply **prey**).

We used the Pierce Co-Immunoprecipitation (Co-IP) Kit #26149 from Termofisher. First, the spin columns are inserted above typical collection tubes (1.5 or 2 ml) (■ Figure 15-B). These tubes collect the volume that passes through the filters of the columns after each centrifugation. The flow-through volume should not exceed ~600 µl when using a 2 ml collection tube and ~300 µl when using a 1.5 ml collection tube to avoid surpassing the column filter, which causes incomplete washing or elution. Antibody-bead covalent coupling is achieved with an amine-reactive resin. We crosslinked 5 µg of antibody to cyanogen bromide-activated resin with sodium cyanoborohydride ($NaBH_3CN$) for 1.5 hours. This step **binds covalently the antibodies** to the A/G beads and prevents them from eluting together with the purified protein complex. Afterwards, the beads were quenched with 1M Tris-HCl supplemented with $NaBH_3CN$ and washed multiple times to remove non-bound antibodies. The principle of protein complex recovery from the sample mixture is represented in ■ Figure 15-C. Sample volumes were normalized to contain 250 µg of protein and were immobilized to the antibody-linked resin with a gentile end-over-end mixing overnight at 4ºC. Non-bound proteins were discarded through multiple spin

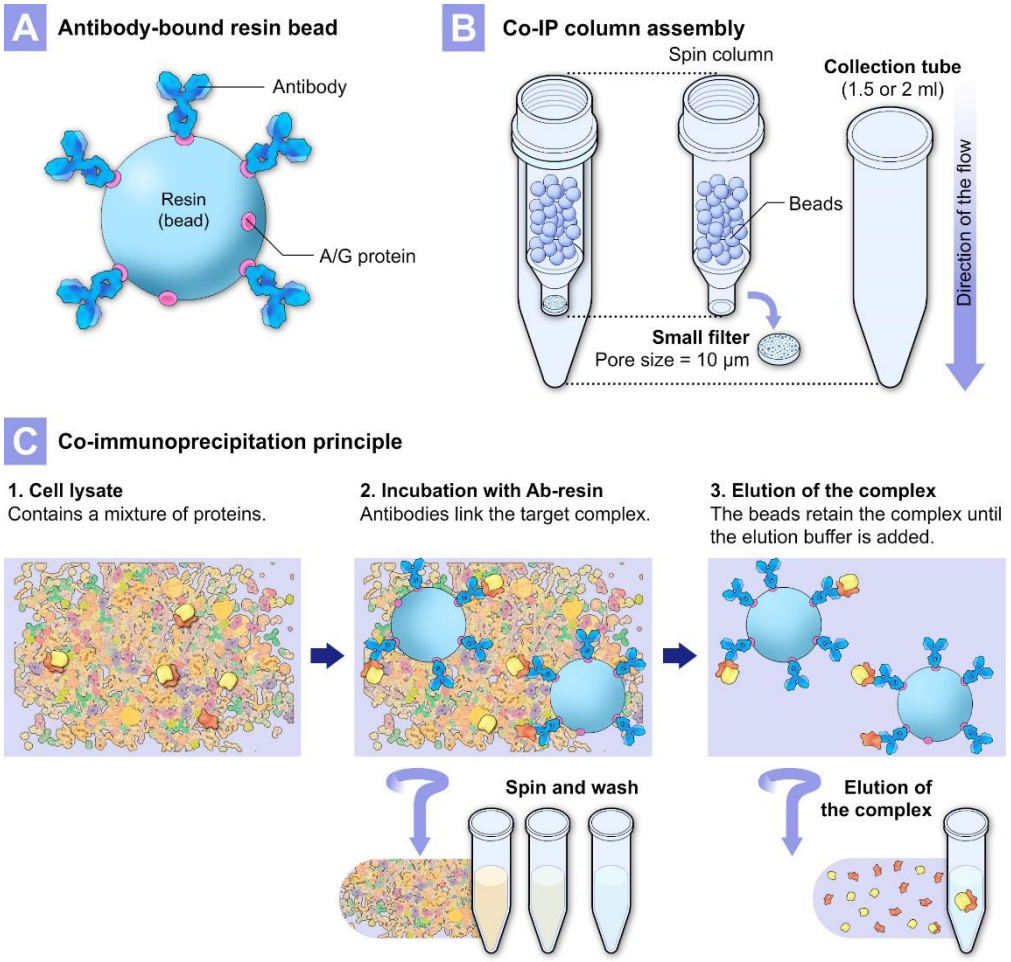

Figure 15. **Co-immunoprecipitation setup.** Co-Immunoprecipitation allows to isolate specific protein complexes from a lysate. A Antibodies are covalently linked to the resin, made of beads, through a covalent bond with A/G protein. B The co-IP assembly consists of a spin column inserted over a collection tube. The spin column contains a small filter with pores of 10 µm in size. C Principle of co-IP. The cell lysate contains an intricate mixture of proteins. When the sample and the antibody-linked resin are incubated together, antibodies bind to the specific protein. Several centrifugation steps wash the resin, eluting away unspecific proteins from the sample. The beads cannot pass through the 10 µm pore and are retained in the column. Finally, an elution wash with an acidic pH separates the complex from the antibody and the target is collected to further analysis. *Source: own elaboration.*

and washing steps and bait-prey complexes were eluted with a low-pH elution buffer and immediately neutralized 1M Tris pH 9.5. In parallel, we performed mock co-IPs to control antibody fragment co-elution (using PBS instead of sample) and to control unspecific protein binding to the beads (using PBS instead of antibody). The eluted complexes were evaluated by SDS-PAGE electrophoresis and Western blotting. None of the negative controls showed immunoreactivity (see Results). The used antibodies (anti-

Cα, anti-Cβ and anti-RIβ) were suitable for IP according to the manufacturer and did not interfere with bait-prey interaction as they were raised against the C-terminal tail of the proteins, which does not participate in R-C subunit interaction.

5.1. Controls for co-IP

Co-Immunoprecipitation can be validated through positive and negative controls (**Table 9**). Analyzing the input sample confirms that the target protein is present before the co-IP and that each immunoprecipitation starts from the same amount of target protein. A possible problem during the elution step is that antibodies can detach from the beads and co-elute with the complex, contaminating the purified sample with the heavy (50 kDa) and light (25 kDa) chains of the antibodies. In Western blot, these fragments can co-migrate with relevant bands, react with the secondary antibody and mask important results. Antibody co-elution can be screened by Western blotting with a negative control for antibodies, where the antibody alone should not elute from the beads nor give signal. To avoid this artifact, the co-IP kit used in this thesis crosslinked antibodies to the beads, generating a covalent bond that minimizes antibody elution. Another problem during co-IP is that agarose beads are porous and unspecific proteins can enter inside and co-elute with the target protein. In Western blot, these proteins can migrate and appear as they were part of the complex with the target protein. Protein attachment to the beads can be screened by Western blotting a negative control for beads, where beads without antibody should not bind any protein nor give any signal in Western blot. This artifact is avoided by increasing the number of centrifugation washes, changing buffer conditions or the material of the beads.

Table 9. Co-immunoprecipitation controls.

Control	Diagram	Interpretation
Normal sample	S-Ab-B	The sample is mixed with antibody-linked beads.
Positive control "Input"	S-( )-( )	The input is used to confirm that the sample contains the target protein before any immunoprecipitation.
Negative control for antibodies	( )-Ab-B	CoIP without sample. Any bands appearing in the Western blot are due to the antibody, which co-elute. Primary antibodies contamination gives a 50 kDa band (heavy chain) and 25 kDa band (light chain).
Negative control for beads	S-( )-B	coIP without antibody. If the target protein appears, it means that the proteins adhere unspecifically to the beads.

6. Statistical analysis

Three animals at least (n≥3) were used as biological replicates for every experiment. All experiments were carried out at least in triplicate and are representative of at least three separate experiments. The results are presented as ratios or percentages of treatment to control (mean ± SEM). We used the Shapiro-Wilk test to test sample normality. Then, a paired Student t-test or its non-parametric alternative Wilcoxon test were used to determine the statistical significance of the ratios (considered as p value <0.05). The calculations were elaborated using R 3.4.3 statistical package.

CHAPTER 4

RESULTS

UNIVERSITAT ROVIRA I VIRGILI
MUSCARINIC RECEPTOR MODULATION OF PROTEIN KINASE A, PROTEIN KINASE C AND EXOCYTOTIC PROTEINS AT THE NEUROMUSCULAR JUNCTION
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Publication 1

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RESEARCH ARTICLE

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The M_2 muscarinic receptor, in association to M_1, regulates the neuromuscular PKA molecular dynamics

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Abstract

Muscarinic acetylcholine receptor 1 subtype (M_1) and muscarinic acetylcholine receptor 2 subtype (M_2) presynaptic muscarinic receptor subtypes increase and decrease, respectively, neurotransmitter release at neuromuscular junctions. M_2 involves protein kinase A (PKA), although the muscarinic regulation to form and inactivate the PKA holoenzyme is unknown. Here, we show that M_2 signaling inhibits PKA by downregulating Cβ subunit, upregulating RIIα/β and liberating RIβ and RIIα to the cytosol. This promotes PKA holoenzyme formation and reduces the phosphorylation of the transmitter release target synaptosome-associated protein 25 and the gene regulator cAMP response element binding. Instead, M_1 signaling, which is downregulated by M_2, opposes to M_2 by recruiting R subunits to the membrane. The M_1 and M_2 reciprocal actions are performed through the anchoring protein A kinase anchor protein 150 as a common node. Interestingly, M_2 modulation on protein expression needs M_1 signaling. Altogether, these results describe the dynamics of PKA subunits upon M_2 muscarinic signaling in basal and under presynaptic nerve activity, uncover a specific involvement of the M_1 receptor and reveal the M_1/M_2 balance to activate PKA to regulate neurotransmission. This provides a molecular mechanism to the PKA holoenzyme formation and inactivation which could be general to other synapses and cellular models.

KEYWORDS

muscarinic receptors, neuromuscular junction, PKA, SNAP-25

Abbreviations: ACh, acetylcholine; AKAP150, A kinase anchor protein 150; AT, atropine; C, catalytic subunit; cAMP, cyclic adenosine monophosphate; co-IP, co-immunoprecipitation; CREB, cAMP response element binding; CSP, cysteine string protein; Cα, catalytic subunit isoform α; Cβ, catalytic subunit isoform β; ExS, extrasynaptic region; H-89, N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide 2 HCl; HRP, horseradish peroxidase; KO, Knockout; M_1, muscarinic acetylcholine receptor 1 subtype; M_2, muscarinic acetylcholine receptor 2 subtype; mAChR, muscarinic acetylcholine receptor; MET, methoctramine; nAChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; PBS, phosphate-buffered saline; PIR, pirenzepine; PKA, protein kinase A; PKC, protein kinase C; R, regulatory subunit; RIα, regulatory type I subunit α; RIβ, regulatory type I subunit β; RIIα, regulatory type II subunit α; RIIβ, regulatory type II subunit β; RIM1α, Rab3 interacting protein 1α; SEM, standard error of the mean; SNAP-25, synaptosome-associated protein 25; α-SNAP, N-ethylmaleimide-sensitive factor attachment protein alpha; Stx, syntaxin; Syn, synaptic region.

Neus Garcia, Josep Maria Tomàs, and Maria Angel Lanuza contributed equally to this study.

1 | INTRODUCTION

Synapses use plastic mechanisms to adjust the strength of the neurotransmitter release to any situation. At the neuromuscular junction (NMJ), muscarinic acetylcholine receptors (mAChR) participate in synaptic plasticity as presynaptic autoreceptors sensing and controlling the release of acetylcholine (ACh).[1-4] The five subtypes of mAChR are classified by their downstream signaling pathway. M_1, M_3, and M_5 use the protein $G\alpha_{q/11}$ to activate phospholipase C and protein kinase C (PKC), whereas M_2 and M_4 use $G\alpha_{i/0}$ to inhibit adenylyl cyclase and protein kinase A (PKA).[5] Even though mammalian NMJ express all five mAChR subtypes,[6] evidence shows that in the adult synapse mainly M_1 and M_2 orchestrate the muscarinic signaling, M_1 increasing ACh release, whereas M_2 decreasing it.[1,4,7]

The PKA pathway has been extensively implicated in synaptic plasticity,[8,9] facilitating the probability of release at many synapses.[10-14] This includes the NMJ, where PKA constitutively promotes normal ACh release.[15,16] In part, PKA could enhance the release by phosphorylating the synaptosome-associated protein-25 (SNAP-25) at Thr^{138},[17,18] which has never been investigated at the NMJ. SNAP-25 phosphorylation by PKA is necessary to maintain the release-ready and primed pool of vesicles.[19] Thus, the PKA phosphorylation of SNAP-25 could be responsible for some effects of muscarinic signaling. In addition, mAChR-PKA role in synaptic plasticity could also be transduced through the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), a master regulator of gene expression, whose activity has been linked to synaptic plasticity at the NMJ as well as in the central nervous system.[20,21]

PKA exists as an inactive tetramer formed by two regulatory (R) and two catalytic (C) subunits which interact among a pseudosubstrate domain. When cAMP binds to the R subunits, the C subunits are liberated and their catalytic activity starts.[22-24] Murine models express four R subunit isoforms (RIα, RIβ, RIIα, RIIβ) and two C subunit isoforms (Cα, Cβ), whereas the Cγ gene is only found in primates.[25,26] Cα and Cβ subunits display essentially the same activation properties, whereas RIβ-containing holoenzymes show increased sensitivity to cAMP-evoked activation than RIα-containing holoenzymes.[27] Also, although RII holoenzymes seem more prone to dissociate than RI, probably due to structural differences, RI are more efficient than RII in inducing CREB response regardless of the C subunit.[28-30]

R subunits are differently expressed across tissues and exert distinct roles in cell differentiation and growth control.[31,32] RIα and RIIα are widely expressed, RIβ is highly expressed in the nervous tissue and RIIβ in the adipose and hepatic tissues.[31-34] Moreover, few studies have located RIα, RIIα and RIIβ at the synaptic area of the skeletal muscle.[35-37] Besides cAMP activation and tissue expression, PKA is also regulated by subcellular targeting.[38,39] In particular, the translocation between cytosol and membrane of both PKA C[40] and R subunits[41,42] regulates their activity. When inactive, most PKA are anchored by binding R subunits to scaffold proteins called A-kinase anchoring proteins (AKAPs). These AKAPs contribute to PKA specificity by recruiting the PKA holoenzyme to distinct subcellular compartments near specific substrates.[43-45] R subunits differ in their subcellular localization, RI being found mainly in the cytosol, whereas RII to the particulate fraction, associated to the nuclei, nucleoli, Golgi complex, and microtubules.[33,46] Additionally, PKA can also be regulated by changes in its concentration through synthesis and degradation[47,48] and by crosstalk with the PKC pathway at the NMJ[16] as well as in other systems[47,49-51] although the molecular signaling involved is unknown.

The present work characterizes at the NMJ how M_1 and M_2 muscarinic signaling regulates the protein expression, subcellular distribution, and membrane-cytosol translocation of specific PKA subunits and whether this regulation extends to the phosphorylation of its targets SNAP-25 and CREB. The main results show that M_2 receptor reduces the Cβ protein level and increases the expression and liberation of R subunits to the cytosol, resulting in the decrease of CREB and SNAP-25 phosphorylation. The downregulation of Cβ also occurs under presynaptic nerve activity. Moreover, a balance M_1/M_2 in the regulation of the PKA is evidenced as some M_2-induced changes need the cooperation of M_1 receptor and also because M_1 receptor produces several changes which oppose to M_2 like recruiting RIα, RIβ, and RIIα to the membrane fraction. Therefore, here we show that M_1 and M_2 muscarinic receptors cooperate at the NMJ to regulate the PKA subunit expression, translocation, and activity on SNAP-25 and CREB phosphorylation. This would be relevant to better understand the molecular regulation of mAChR-PKA signaling over the neurotransmitter release and synaptic plasticity mechanisms.

2 | MATERIALS AND METHODS

2.1 | Animal care

The animals were cared for in accordance with the European Community Council Directive guidelines for the humane treatment of laboratory animals. Male and female adult Sprague Dawley rats (40-80 days; Criffa, Barcelona, Spain; RRID:RGD_5508397) were euthanized for tissue harvest and analysis. No sex-related differences were found when studying the treatments presented in this work. At least three animals ($n \geq 3$) were used as biological replicates for every experiment detailed below. All animal work was approved by the Ethics Comitee of Animal Experimentation of the Universitat Rovira i Virgili.

2.2 | Antibodies

The antibodies used for western blotting and their dilution are listed in Table 1. These antibodies presented bands of the predicted molecular weight without near prominent unspecific bands. The anti-M_1 and anti-M_2 antibodies were tested by subcellular fractionation and antigen preincubation. The corresponding bands of these GPCRs were enriched in the membrane fraction, absent in the cytosolic fraction and antigen preincubation strongly reduced them. The specificity of anti-SNAP-25 antibody was carefully determined in Simó et al, 2019 and we tested the recognition of its cAMP-dependent phosphorylation by anti-pSNAP-25 Thr^{138} antibody with H-89.

2.3 | Knockout cell lines

Anti-PKA antibodies were validated against knockout lysates to guarantee discrimination between isoforms. We used the following CRISPR/Cas-9 knockout controls: Cα knockout 293T cell lysate (#NBP2-65840), Cβ knockout HeLa cell lysate (#NBP2-64806), RIα knockout 293T cell lysate (#NBP2-65698), and RIIα knockout HeLa cell lysate (#NBP2-65316).

2.4 | Chemicals

2.4.1 | Muscarinic inhibition

Pirenzepine dihydrochloride (Tocris): 10 mM stock and used at 10 μM. Methoctramine tetrahydrochloride (Sigma): 1 mM stock and used at 1 μM. Atropine (Sigma): 200 μM stock and used at 2 μM.[1]

2.4.2 | PKA inhibition

PKA activity was blocked with N-[2-((*p*-Bromocinnamyl) amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89, Calbiochem). H-89 was made as 5 mM stock and used at 5 μM.

In the experiments involving nerve stimulation treatment, muscle contraction was blocked using μ-conotoxin GIIIB (#C-270, Alomone Labs Ltd, Jerusalem, Israel). This toxin selectively inhibits sarcolemmal voltage-dependent sodium channels (VDSCs) without affecting synaptic ACh release and has been used extensively for electrophysiology studies at the diaphragm.[16,52-54] It was supplied as lyophilizesd powder of >99% purity and used at 1.5 μM.

All chemicals were diluted in Ringer's solution and both control and drug-containing solutions contained 0.1% dimethyl sulfoxide (DMSO) as the vehicle.

2.5 | Tissue dissection and treatment

Diaphragm muscles from adult Sprague Dawley rats was dissected with special care to preserve phrenic nerve connectivity. Isolated nerve-muscle preparations were immersed in Ringer's solution (mM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 12, glucose 12.1, and DMSO 0.1%), oxygenated with O_2:CO_2 (95:5), and maintained at 26°C.

TABLE 1 Primary antibodies

Target	Epitope	Source	Company (#cat)	Dilution
M_1 mAChR	Hu M_1 mAChR residues 227-353	Ms mAb	Alomone (AMR-001)	1/2000
M_2 mAChR	Hu M_2 mAChR residues 168-192	Ms mAb	Abcam (ab90805)	1/2000
Cα	Hu Cα C-terminus	Rb pAb	Santa Cruz (sc-903)	1/1000
Cβ	Hu Cβ C-terminus	Rb pAb	Santa Cruz (sc-904)	1/1000
RIα	Hu RIα residues 1-381	Ms mAb	Santa Cruz (sc-136231)	1/1000
RIβ	Hu RIβ C-terminus	Rb pAb	Santa Cruz (sc-907)	1/1000
RIIα	Ms RIIα C-terminus	Rb pAb	Santa Cruz (sc-909)	1/1000
RIIβ	Hu RIIβ residues 21-110	Ms mAb	Santa Cruz (sc-376778)	1/1000
CREB	Hu CREB synthetic peptide	Rb pAb	CST (9192)	1/1000
pCREB (Ser^{133})	Hu CREB residues around $pSer^{133}$	Rb pAb	CST (9191S)	1/1000
SNAP-25	Hu SNAP-25 residues around Gln^{116}	Rb mAb	CST (5309)	1/1000
pSNAP-25 (Thr^{138})	Hu SNAP-25 residues around Thr^{138}	Rb pAb	Biorbyt (orb163730)	1/1000
AKAP150	Rat AKAP150 residues 428-449	Rb pAb	Millipore (07-210)	1/1000
Na/K ATPase	Chicken ATPase residues 27-55	Ms mAb	DSHB (a6f)	1/2000
GAPDH	Rb GAPDH	Ms mAb	Santa Cruz (sc-32233)	1/2000

Note: Antibodies used in this study and procedure specifications.

Abbreviations: Dk, donkey; Hu, human; mAb, monoclonal antibody; Ms, mouse; pAb, polyclonal antibody; Rb, rabbit.

All treatments were performed ex vivo on excised diaphragm muscles. The experimental design was pairwise: one hemidiaphragm underwent the treatment, while the other served as its paired untreated control. Single-inhibitor treatments were applied for 60 minutes, a period which allows the comparison with previous studies in the same model.[55-57] Double-inhibitor treatments were performed to study the implication of PKA in muscarinic signaling. In these, the treated hemidiaphragms were first preincubated for 30 minutes in Ringer solution containing H-89 and afterward for further 30 minutes in Ringer solution containing H-89 plus the muscarinic inhibitor specified. The controls of the doubly inhibited preparations were incubated for 60 minutes in Ringer solution containing H-89 to discard the effects of the PKA inhibitor.

2.6 | Phrenic nerve stimulation

In some experiments, we studied the muscarinic signaling under the presence of centrifugal input, that is, with phrenic nerve stimulation (previously described in 58). In these experiments, the diaphragm was stimulated through the phrenic nerve with a pulse generator (CIBERTEC Stimulator CS 20) linked to a stimulus isolation unit (CIBERTEC ISU 165). Visible contractions served to verify successful nerve dissection before applying the contraction blocker μ-conotoxin GIIIB. After the preincubation of μ-conotoxin GIIIB (30 minutes), phrenic nerves were stimulated at 1 Hz for 30 minutes, a protocol which allows the maintenance of tonic functions without depleting synaptic vesicles. To study muscarinic signaling, treated hemidiaphragm preparations contained muscarinic inhibitors during the preincubation and stimulation, whereas control preparations did not contain muscarinic inhibitors.

2.7 | Sample processing and fractionation

2.7.1 | Whole cell lysates

After being treated as indicated, the muscles were immediately frozen in liquid nitrogen. Homogenization was performed with an overhead stirrer (VWR International, Clarksburg, MD) in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl 50 (pH 7.4), EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; NP-40 1%, Triton X-100 0.1%, and protease inhibitor cocktail 1% (Sigma, Saint Louis, MO, USA)). Insoluble materials were removed with two centrifugations at 4°C: 1000*g* for 10 minutes and 15 000*g* for 20 minutes. The final supernatant contained the whole cell fraction lysate.

2.7.2 | Membrane/cytosol fractionated lysates

Unlike whole cell lysates, for membrane-cytosol fractionation, we homogenized the samples immediately after treatment (without a freezing step) and with a detergent-free lysis buffer (in mM: NaCl 150, Tris-HCl 50 (pH 7.4), EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1, and protease inhibitor cocktail 1%). Insoluble materials were removed by centrifugation at 1000*g* for 15 minutes at 4°C. The resulting supernatant was further centrifuged at 130 000*g* for 1 hour. The new supernatant corresponded to the cytosolic fraction, while the pellet to the membrane fraction. The latter was resuspended in detergent-containing lysis buffer (see above). The purity of the subcellular fractionation was validated by western blotting of the fraction-specific proteins GAPDH for cytosol and Na^+/K^+-ATPase for membrane.

2.7.3 | Synaptic/Extrasynaptic fractionated lysates

After treating the muscles, we separated the synaptic and extrasynaptic regions as indicated in Figure 6A and previously stated.[59] We performed control experiments to check that our separation protocol was accurate by incubating some diaphragms with TRITC conjugated α-bungarotoxin and staining the nerves with an antibody against antineurofilament-200. We did not detect any nerve nor postsynaptic area in the extra-synaptic region. Once separated, synaptic and extrasynaptic regions were processed equally.

2.8 | Western blotting

The protein content of the samples was determined by the DC protein assay (Bio-Rad, CA, USA). Sample volumes containing 30 μg of protein were loaded in an 8% SDS-PAGE gel (10% to detect SNAP-25), separated for 10 min at 90V and 60 min at 110 V, and electrotransferred to PVDF membranes (Bio-Rad, CA, USA). Blocking solutions were TBST containing 5% nonfat dry milk or 5% BSA. The primary antibodies were incubated overnight at 4°C and the HRP-conjugated secondary antibodies for 1 hour. Chemiluminescence was revealed with an ECL kit (GE Healthcare Life Sciences, UK) and imagined with the ChemiDoc XRS+ Imaging System (Bio-Rad, CA, USA).

ImageJ software was used to calculate the optical density of the bands, always from the same immunoblot image. The values were normalized to (a) the background values and (b) the total protein transferred on the PVDF membranes,

analyzed with Sypro Ruby protein blot stain, (Bio-Rad, CA, USA).[60] Ratios between the experimental and control were calculated from the same membrane image. All presented data derive from densitometry measurements made of 3-10 separate replicates, plotted against controls. For western blot desing no blinding was performed. Data quantification was performed blindly.

2.9 | Immunohistochemistry

The PKA Cβ subunit was localized at the NMJ of diaphragm and *levator auris longus* (LAL) muscles by immunohistochemistry with identical results. The thinness of LAL muscles improved the imaging and analysis of NMJs. Whole muscle mounts were fixed with 4% paraformaldehyde for 30 minutes. After fixation, the muscles were rinsed with PBS and incubated in 0.1 M glycine in PBS. Afterward, PBS supplemented with 1% Triton X-100 and 4% BSA was used for permeabilization and blockade of nonspecific binding. Then, muscles were incubated overnight at 4°C in a mixture of primary antibodies raised in different species (anti-Cβ subunit and anti-S100 to label Schwann cells) and then rinsed. The muscles were then incubated for 4 hours at room temperature in a mixture of appropriate secondary antibodies. AChRs were detected with α-BTX conjugated with TRITC. At least three muscles were used as negative controls and no cross-reaction was detected between antibodies. In some muscles, plastic embedded semithin sections (0.5 μm) were obtained for high-resolution immunofluorescence analysis of the neuromuscular junction molecules as previously described.[61] Immunolabeled NMJs from the whole-mount muscles were viewed with a laser-scanning confocal microscope (Nikon TE2000-E). Special consideration was given to the possible contamination of one channel by another. In experiments involving negative controls, the photomultiplier tube gains and black levels were identical to those used for a labeled preparation made in parallel with the control preparations. Images were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA) and neither the contrast nor brightness were modified.

2.10 | Co-immunoprecipitation

Co-immunoprecipitation was performed with the Pierce Co-IP kit (Thermo Scientific, USA). Briefly, 5 μg of antibody were crosslinked to cyanogen bromide-activated resin with sodium cyanoborohydride ($NaBH_3CN$) for 1.5 hours. Afterward, the beads were quenched with 1 M Tris HCl supplemented with $NaBH_3CN$ and washed multiple times to remove nonbound antibodies. Sample volumes were normalized to contain 250 μg of protein and were immobilized to the appropriate antibody-resin with a gentile end-over-end mixing overnight at 4°C. Nonbound proteins were discarded through multiple washing steps and bait-prey complexes were eluted with a low-pH elution buffer and immediately neutralized 1 M Tris pH 9.5. In parallel, we performed mock co-IPs to control antibody fragment co-elution (using PBS instead of sample) and to control unspecific protein binding to the beads (using PBS instead of antibody). The eluted complexes were evaluated by SDS-PAGE and western blotting. None of the negative controls showed immunoreactivity (see Results). The used antibodies (anti-Cα, anti-Cβ, and anti-RIβ) were suitable for IP according to the manufacturer and did not interfere with bait-prey interaction as they were raised against the C-terminal tail of the proteins, which does not participate in R-C subunit interaction.

2.11 | Statistical analysis

The sample size was calculated as described in.[62] All experiments were carried out at least in triplicate and are representative of at least three separate experiments. The results are presented as ratios or percentages of treatment to control (mean ± SEM). Shapiro-Wilk test was used to test sample normality. Paired Student t test or its nonparametric alternative Wilcoxon test were used to determine the statistical significance of the ratios (considered as P value $<.05$). The calculations were elaborated using R 3.4.3 statistical package.

3 | RESULTS

3.1 | M_2 inhibits M_1 protein levels

To study the interplay between mAChR subtypes, we asked whether the inhibition of M_1 and M_2 affected their own and mutual expression. Selective muscarinic inhibition was carried out with the M_1-inhibitor pirenzepine (PIR), the M_2-inhibitor methoctramine (MET) and the M_1/M_2 pan-inhibitor atropine (AT). Figure 1A shows that M_1 blockade did not affect the protein levels of any receptor (M_1: 0.8 ± 0.1; M_2: 1.0 ± 0.2). On the contrary, M_2 blockade increased the levels of M_1 receptor (1.7 ± 0.2) without altering the own M_2 (1.0 ± 0.1). This indicates that M_2 signaling constitutively reduces M_1 levels. Additionally, M_1/M_2 inhibition (AT) did not modify any receptor (M_1: 0.8 ± 0.1; M_2: 1.0 ± 0.1). The difference between the effects of MET and AT suggests that M_2 blockade needs M_1 mAChR activity. We also identified this M_2 regulation pattern dependent of M_1 active in other findings of this work

involving PKA subunits regulation (see the following sections). In addition to AT, we used a mixture of PIR and MET (PIR+MET) to also check the implication of both M_1 and M_2 mAChRs. This muscarinic blockade is more selective for M_1 and M_2 than AT and reduces the likelihood of alteration of other mAChR subtypes. Although expressed at the adult NMJ, M_3 and especially M_4 subtypes only participate in the development of newborn NMJ,[63-65] and only M_1 and M_2 subtypes orchestrate the release at the adult NMJ.[2,63,66] As expected, we found that PIR+MET mimicked the effect of atropine in all the conditions tested, reinforcing the participation of solely the M_1 and M_2 mAChR subtypes. This in line with reports showing that the mixture of PIR+MET fully mimicks the effect of atropine on ACh release at the frog NMJ and also mimicks the effect of the pan-muscarinic inhibitor scopolamine.[4,67]

3.2 | Muscarinic modulation of PKA catalytic and regulatory subunits protein levels

To study the protein levels of the PKA C and R subunits, we selected the antibodies which showed high specificity for the corresponding protein at the predicted molecular weight (in kDa): Cα 40, Cβ 40, RIα 48, RIβ 51, RIIα 50, and RIIβ 53 in the rat diaphragm (Figure 2A,B).[68,69] Antibody specificity was validated through knockout cell lysates (Figure 2A). The anti-Cα antibody was reactive against the control and Cβ-KO lysates but negative against the Cα-KO. Inversely, anti-Cβ antibody was reactive against the control and Cα-KO lysates but negative against the Cβ-KO. This confirmed that anti-Cα and -Cβ antibodies do not cross-react between C subunits. Regarding the anti-RIα and -RIIα antibodies, their ~50 kDa band was positive in the control, abolished in their respective KO cells and unaffected by the KO of the other R subunit. To our knowledge, no RIβ-KO and RIIβ-KO cells are commercially available to perform a similar validation on the corresponding antibodies. Alternatively, antibodies can be validated with cell lines which do not express the target protein. We found no detectable levels of RIβ and RIIβ in the 293T and HeLa cell lysates, consistent with reports of no detectable expression in these cell lines[28] and minimal expression in comparable human tissues.[32] Furthermore, multiple sequence alignment with Clustal2.1 indicated that the antigens of anti-RIβ and -RIIβ antibodies are identical to the corresponding subunits and disparate from the other PKA subunits. Some anti-R antibodies showed an unspecific 30-kDa band which did not correspond to the predicted molecular weight and was not affected by any KO.

Once the anti-PKA antibodies were validated, we studied whether muscarinic signaling regulates the protein levels of C and R subunits. M_1 inhibition (Figure 2C) decreased the protein levels of the regulatory subunit RIIβ (0.6 ± 0.1), without affecting any other PKA subunit (Cα 1.2 ± 0.1, Cβ 1.1 ± 0.1, RIα 1.0 ± 0.1, RIβ 1.2 ± 0.2, and RIIα 1.3 ± 0.1). This suggests that M_1 pathway could constitutively reduce PKA activity through the increase of RIIβ levels. M_2 inhibition (Figure 2D) caused a twofold increase in Cβ (1.9 ± 0.4) without altering Cα (1.2 ± 0.1). In addition, M_2 blockade decreased the regulatory subunits RIIα (0.7 ± 0.1) and RIIβ (0.7 ± 0.1), but not RIα or RIβ (respectively: 1.0 ± 0.02 and 1.1 ± 0.2). The downregulation of Cβ and upregulation of RIIα and RIIβ is consistent with the well-known role of M_2 signaling as inhibitor of PKA activity. M_1/M_2 inhibition with atropine (Figure 2E) or a mixture of PIR and MET (Figure 2F) only reduced the protein levels of RIIβ (0.8 ± 0.04), while the other subunits remained unchanged after the treatment. RIIβ downregulation could be linked to the activity of both M_1 and M_2

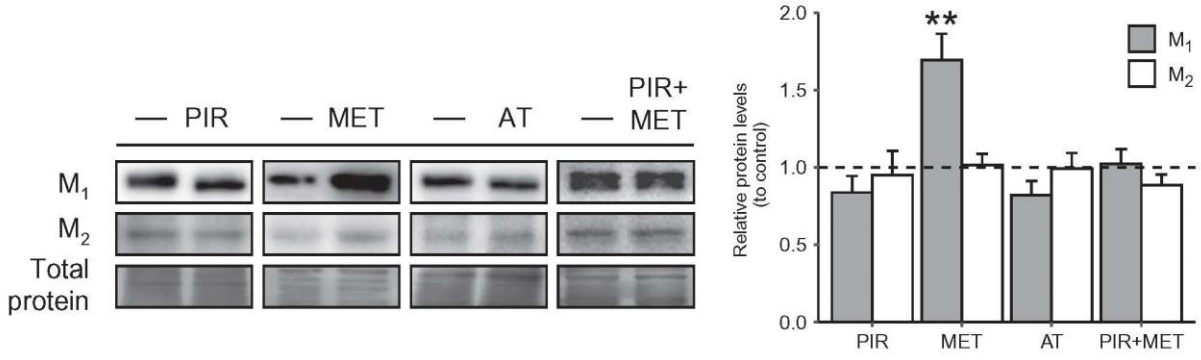

FIGURE 1 M_2 signaling inhibits the protein levels of M_1. Representative western blot and data quantification of M_1 and M_2 protein levels in the diaphragm muscle after M_1-inhibition (PIR, 10 μM), M_2-inhibition (MET, 1 μM), and M_1/M_2 inhibition by atropine (AT, 2 μM) and by a mixture of PIR and MET (PIR+MET; PIR, 10 μM + MET, 1 μM). Data are fold changes vs basal condition values: mean ± SEM. $^{**}P < .01$ n = 3; >5 repeats per n. AT, atropine; M_1, muscarinic receptor subtype 1; M_2, muscarinic receptor subtype 2; MET, methoctramine; nAChR, nicotinic acetylcholine receptor; PIR, pirenzepine; Stx, syntaxin

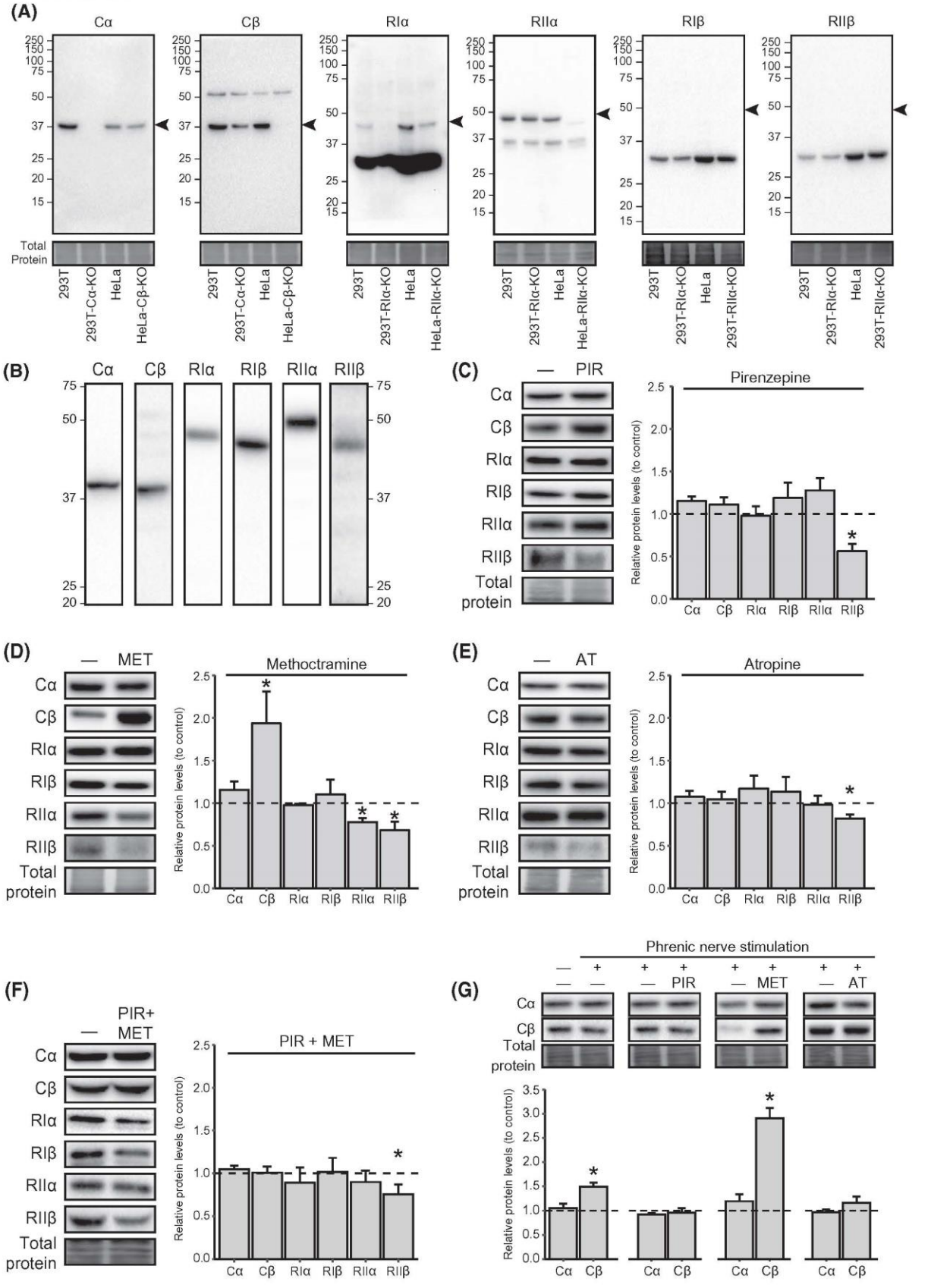
(A)
Cα
Cβ
RIα
RIIα
RIβ
RIIβ
Total Protein
293T
293T-Cα-KO
HeLa
HeLa-Cβ-KO
293T-RIα-KO
HeLa-RIIα-KO
293T-RIIα-KO
(B)
(C)
— PIR
Pirenzepine
Total protein
Relative protein levels (to control)
(D)
— MET
Methoctramine
(E)
— AT
Atropine
(F)
PIR+ MET
PIR + MET
(G)
Phrenic nerve stimulation
— PIR
— MET
— AT

FIGURE 2 Muscarinic signaling modulates PKA C and R subunits protein levels. A, Knockout validation of the specificity of the anti-Cα, -Cβ, -RIα, -RIβ, -RIIα, and -RIIβ antibodies by immunoblotting. B, Western blot analysis of diaphragm samples showing that bands are consistent with their predicted molecular weight. C-G, Western blot analysis and data quantification of PKA C and R protein levels in the diaphragm muscle after (C) M_1-inhibition (pirenzepine, 10 μM), (D) M_2-inhibition (methoctramine, 1 μM) and (E) M_1/M_2 inhibition by atropine (AT, 2 μM), and (F) by a mixture of pirenzepine and methoctramine (PIR+MET; PIR, 10 μM + MET, 1 μM). G, Western blot analysis and data quantification of PKA Cα and Cβ protein levels in the diaphragm muscle under nerve stimulation. Data are expressed as fold change vs basal condition values: mean ± SEM. *$P < .05$; n = 3; >5 repeats per n. AT, atropine; Cα/β, protein kinase A catalytic subunit α/β; MET, methoctramine; PIR, pirenzepine; RIα/RIβ/RIIα/RIIβ, protein kinase A regulatory subunit Iα/Iβ/IIα/IIβ

subtypes, because their selective inhibition induced the same effect. Interestingly, the pan-inhibitor AT and the mixture PIR+MET did not mimick the effect of MET on Cβ and RIIα. Similarly to the M_1 downregulation, this is another node where M_2 effect needs M_1 active. Finally, we also studied whether the muscarinic modulation of catalytic subunits in presence of cholinergic input from phrenic nerve (Figure 2G). Phrenic nerve stimulation per se did not affect the levels of Cα (1.0 ± 0.1) and increased those of Cβ (1.5 ± 0.1). Similarly to basal conditions, M_2 blockade increased Cβ levels (2.9 ± 0.2) but not M_1 or M_1/M_2 inhibition (respectively: 1.0 ± 0.1; 1.2 ± 0.1). This indicates that M_2 mAChR signaling opposes to the Cβ-enhancing action of nerve stimulation.

In summary, both M_1 and M_2 receptors constitutively promote increased levels of RIIβ protein, which suggests some impairment of PKA activity. In comparison with M_1, M_2 would further reduce PKA activity by decreasing Cβ and increasing RIIα, actions which need the activity of M_1.

3.3 | Regulation of cytosol-membrane PKA subunits translocation by mAChR

Because neurotransmission relies on membrane trafficking and PKA action is regulated by subcellular distribution, we next examined how muscarinic signaling redistributes the PKA subunits between the membrane and cytosol fraction. Figure 3 shows the percentage of each PKA subunit in the cytosol and membrane fraction before and after muscarinic blockade in the diaphragm muscle. Line plots represent the cytosol/total ratio (ie, both control and treatment values defined as 100%), whereas bars represent the relative percentages (ie, control defined as 100% and treatment calculated in relation to control); the last is only discussed when protein levels change. Additionally, all data were normalized to the total protein loaded. We used Na^+/K^+-ATPase and GAPDH as markers to confirm the purity of the subcellular fractionation. Na^+/K^+-ATPase and GAPDH were highly enriched in their fraction and essentially undetectable in the counterwise. Both C subunits were predominantly located in the cytosol fraction (% cytosol/total: Cα 79.8 ± 0.02; Cβ 78.7 ± 1.6) although also identifiable in the membrane (Figure 3A-C). Muscarinic imbalance did not induce the translocation of any C subunit. Similarly, in basal conditions, all R subunits were predominantly found in the cytosol (% cytosol/total: RIα 74.7 ± 3.2, RIβ 75.7 ± 7.6, RIIα 57.8 ± 4.4, and RIIβ 71.3 ± 3.2; Figure 3A-C). In line with previous studies, we found RII type subunits more linked to the membrane fraction than RI type.[33,46] Contrary to C subunits, muscarinic signaling modulated the location of R subunits. Particularly, M_1 blockade translocated RIα, RIβ, and RIIα from the membrane to the cytosol (% cytosol/total: RIα +7.4 ± 2.7; RIβ +11.8 ± 2.8; RIIα +11.9 ± 4.8) (Figure 3A). Pirenzepine decreased RIIβ protein levels in the cytosol and membrane (% cytosol: −29.3 ± 7.3; % membrane: −10.4 ± 2.9) without changing the translocation ratio between these compartments (% cytosol/total −5.1 ± 2.9). Conversely, M_2 blockade translocated RIβ and RIIα from the cytosol to the membrane (% cytosol/total; RIβ −9.5 ± 4.5; RIIα −30.4 ± 6.0) without affecting RIα and RIIβ (% cytosol/total; RIα −2.9 ± 2.4; RIIβ −4.5 ± 3.4) (Figure 3B). The treatment with the pan-inhibitor AT and the mixture PIR+MET did not change any regulatory subunit position, suggesting that the operativity of both receptors is needed to accomplish the membrane-cytosol translocation events (Figure 3C).

After determining that muscarinic imbalance affects the association of PKA to the cytosol and membrane compartments, we wondered whether their anchor protein AKAP150 could be involved because it participates in neuronal processes and muscarinic signaling.[70] We used an anti-AKAP150 antibody which reacted with a unique band of the predicted 150 kDa molecular weight (Figure 4A). This antibody was raised against the peptide sequence corresponding to the amino acids 428-449 of rat AKAP150. Blasting this sequence against a rat database showed 100% identity with AKAP150 (Uniprot sequence P24587), whereas the other hits presented gaps, less than 60% identity and their molecular weight did not correspond to the observed band (40-86 kDa vs the observed 150 kDa). In the total fraction (Figure 4B), M_1 inhibition decreased AKAP150 protein levels (0.3 ± 0.1), whereas M_2 inhibition increased them (1.4 ± 0.2). Probably due to their balance, M_1/M_2 inhibition did not induce any change (1.1 ± 0.2). When analyzing membrane and cytosol fractions, we found that AKAP150 is majorly located in the membrane in basal conditions (membrane/total: AKAP150 88.2% ± 6.3) (Figure 4C). In concordance with the previous results, the pirenzepine-induced

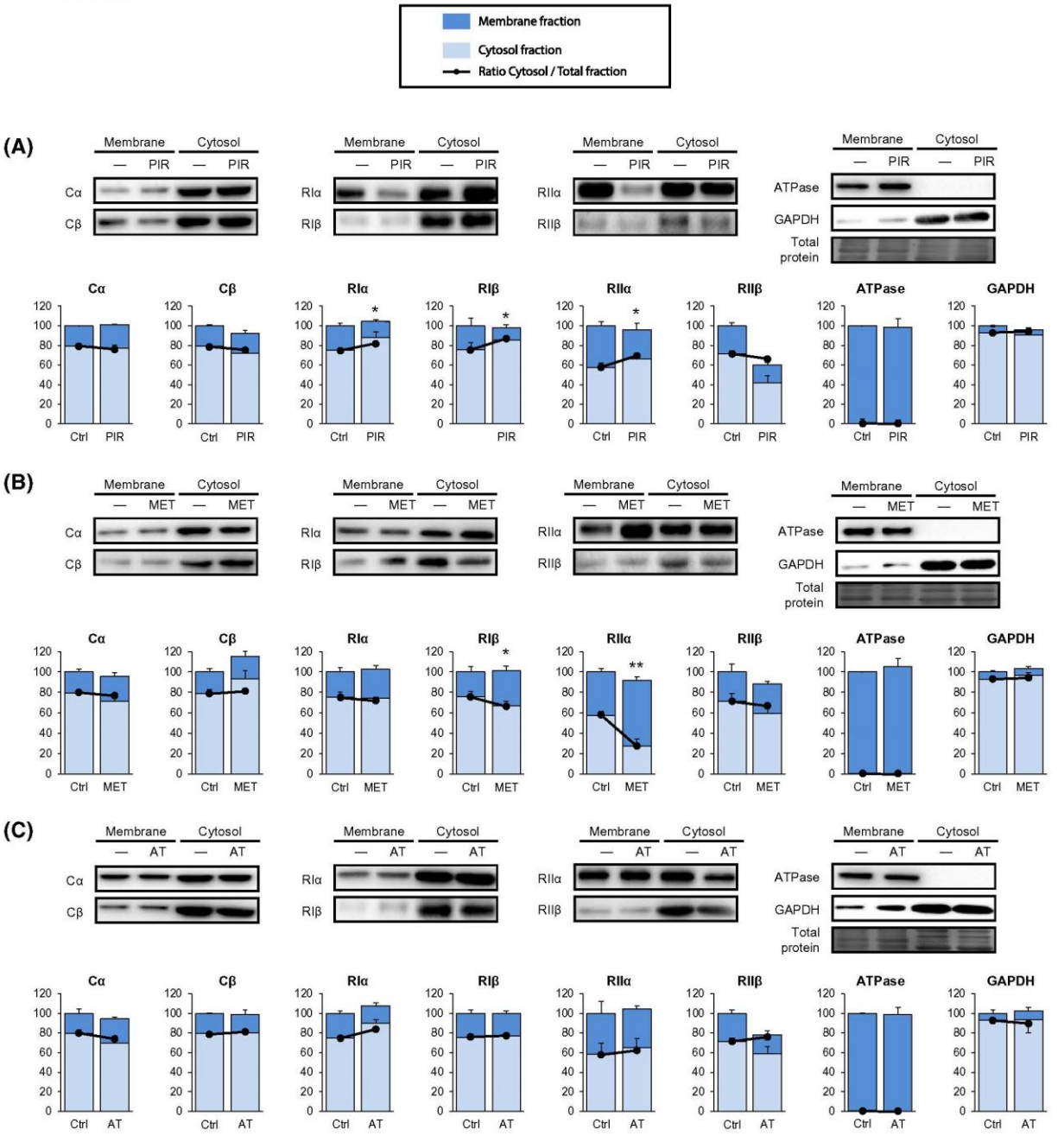

FIGURE 3 mAChR modulate the cytosol/membrane translocation of PKA R subunits. Western blot analysis and data quantification of the protein levels of PKA Cα, Cβ, RIα, RIβ, RIIα, RIIβ, ATPase, and GAPDH in the membrane and cytosol fractions of the diaphragm muscle after (A) M_1-inhibition (pirenzepine, 10 μM), (B) M_2-inhibition (methoctramine, 1 μM), and (C) M_1/M_2 inhibition by atropine (AT, 2 μM) and by a mixture of PIR and MET (PIR+MET; PIR, 10 μM + MET, 1 μM). Data in line plots are percentages of immunoreactivity in the cytosol vs immunoreactivity in the cytosol and membrane (% cytosol/total). Data in bars represent the percentages of immunoreactivity in the cytosol and membrane (ie, control defined as 100% and treatment calculated in relation to control). All data have been normalized to the total amount of loaded protein: mean ± SEM. $*P < .05$ $**P < .01$; n = 3; >5 repeats per n. AT, atropine; Cα/β, protein kinase A catalytic subunit α/β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MET, methoctramine; PIR, pirenzepine; RIα/Iβ/IIα/IIβ, protein kinase A regulatory subunit Iα/Iβ/IIα/Iiβ

decrease of RIα, RIβ, and RIIα in the membrane fraction was accompanied by a significant decrease in its anchoring protein AKAP150 in the same fraction (% membrane: −67.1% ± 4.8). The methoctramine-induced increase in RIβ and RIIα protein levels in the membrane fraction was accompanied by a significant increase of AKAP150 in the same

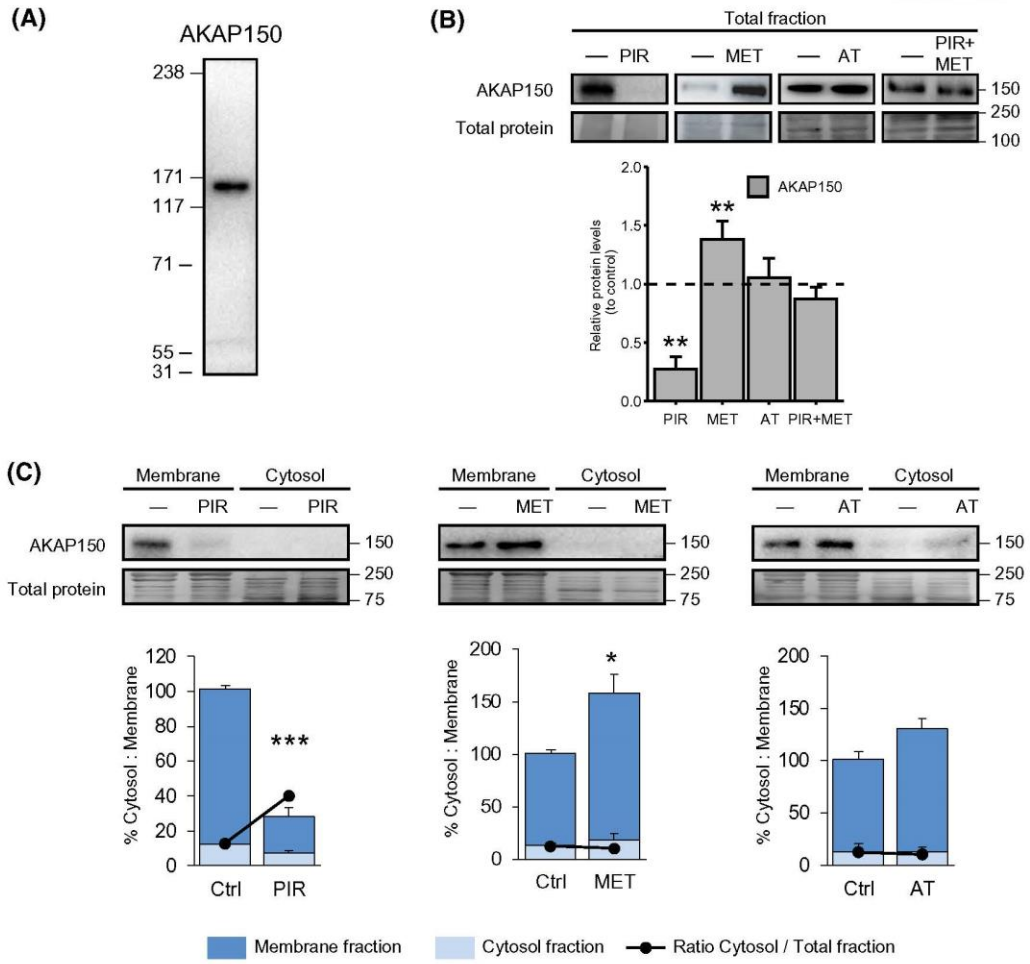

FIGURE 4 Both M_1 and M_2 modulate AKAP150 protein levels. A, Representative band of the anti-AKAP150 antibody at the diaphragm showing its corresponding 150kDa band. B, Western blot analysis and data quantification of AKAP150 protein levels in the diaphragm muscle after M_1-inhibition (PIR), M_2-inhibition (MET) and M_1/M_2-inhibition (AT and PIR+MET). Data are fold change vs basal condition values: mean ± SEM. C, Western blot analysis and data quantification of AKAP150 in the membrane and cytosol fractions of the diaphragm. Data in line plots are percentages of immunoreactivity in the cytosol vs cytosol and membrane (% cytosol/total). Data in bars represent the percentages of immunoreactivity in the cytosol and membrane (ie, control defined as 100% and treatment calculated in relation to control). All data have been normalized to the total amount of loaded protein: $*P < .05$ $**P < .01$ $***P < .001$; n = 3; >5 repeats per n. AKAP150, A kinase anchor protein 150; AT, atropine; MET, methoctramine; PIR, pirenzepine

fraction (% membrane: +51.2 ± 18.3). Finally, atropine did not change AKAP150 in the membrane fraction in concordance with the previous results in the total fraction (% membrane: +28.7 ± 9.6).

In summary, mAChRs modulate the cytosol-membrane translocation of PKA regulatory rather than catalytic subunits. The constitutive action of M_1 receptor seems to recruit RIα, RIβ, and RIIα to the membrane fraction whereas the constitutive effect of M_2 mAChR might inhibit the action of M_1 and release RIβ and RIIα to the cytosol, increasing the probability to assemble the holoenzyme. This balance involves the anchoring protein AKAP150, whose levels are regulated to recruit PKA subunits or release them to the cytosol.

3.4 | M_2 regulates the interaction between synaptic-enriched PKA subunits

To further prove if the translocation of R subunits to the membrane was accompanied by a release of the cytosolic C subunits, we investigated their interaction after M_2 inhibition (Figure 5). Due to the wide cellular and tissue distribution of PKA subunits, we detected them in the synaptic (Syn) and extrasynaptic (ExS) regions (Figure 5A). We studied Cα and Cβ and focused on their relation with RIβ because (a) its levels do not change after muscarinic inhibition, but (b) it translocates after treatment possibly modulating the cytosolic C subunits and (c) it is specifically expressed in the nervous system.[31-34] Figure 5B shows that Cβ and RIβ are enriched in the synaptic

area of nontreated diaphragms (Cβ: 1 Syn; 0.6 ± 0.1 ExS; RIβ: 1 Syn; 0.4 ± 0.03 ExS), while Cα was almost equally distributed between synaptic and extrasynaptic (Cα: 1 Syn; 0.96 ± 0.02 ExS). In concordance with the results in the total lysate (see Figure 2), the treatment with MET increased Cβ in both synaptic and extrasynaptic regions (Cβ: +0.2 ± 0.2 Syn+MET; +0.2 ± 0.1 ExS+MET) without changing RIβ (RIβ: −0.09 ± 0.1 Syn+MET; +0.01 ± 0.1 ExS+MET).

Next, to precisely locate Cβ in the NMJ, we used fluorescent immunohistochemistry and confocal microscopy. Plastic-embedded semithin sections (0.5 μm) were used for high-resolution immunofluorescence analysis of neuromuscular junction molecules.[61] Figure 5C shows a NMJ stained with triple labeling: Cβ in green, AChRs in red, and Schwann cells (S100) in blue. The PKA Cβ subunit label is clearly present in granular form on the S100-positive teloglial cells. The muscle cell also presents a very faint general labeling on the sarcoplasm, which is higher in a band (arrow) around 2 μm below the AChR-delineated postsynaptic gutters. Also, remarkable Cβ labeling can be observed in the space occupied by the nerve terminal between the blue S100-positive Schwann cell and the red postsynaptic gutters (arrowhead). In

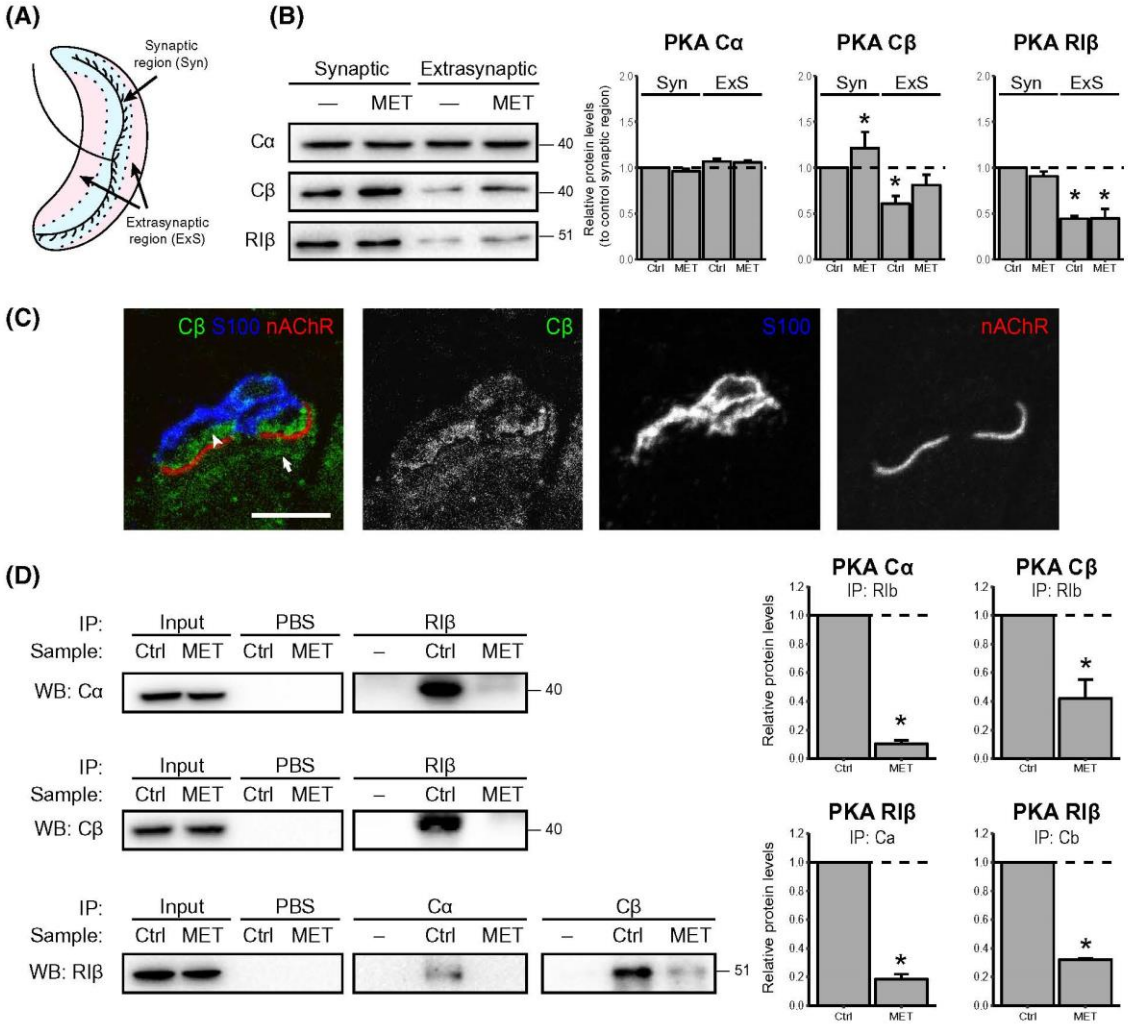

FIGURE 5 M_2 increases the interaction of PKA Cα and Cβ with the synaptic region enriched-RIβ. A, Schematic representation of the synaptic and extrasynaptic regions of the rat diaphragm. B, Western blot analysis and data quantification of the protein levels of Cα, Cβ, and RIβ in the synaptic and extrasynaptic regions of the diaphragm before and after M_2-inhibition (methoctramine, 1 μM). C, Plastic-embedded semithin sections (0.5 μm) of the neuromuscular junctions of LAL muscle visualized at the confocal microscope. NMJ with triple labeling: Cβ in green, S100 in blue, and nicotinic AChRs in red. Cβ is present in the three cell components of the neuromuscular synapse. Scale bars = 10 μm. D, Co-immunoprecipitation analysis and data quantification of the effect of M_2 inhibition on the interaction between the RIβ with Cα and Cβ. Data are expressed as fold change vs basal condition: mean ± SEM. The signal for the immunoprecipitated protein has been normalized to that in the input. *$P < .05$; n = 3; >5 repeats per n. Cα/β, protein kinase A catalytic subunit α/β; MET, methoctramine; nAChR, nicotinic acetylcholine receptors; PIR, pirenzepine; RIβ, protein kinase A regulatory subunit Iβ [Color figure can be viewed at wileyonlinelibrary.com]

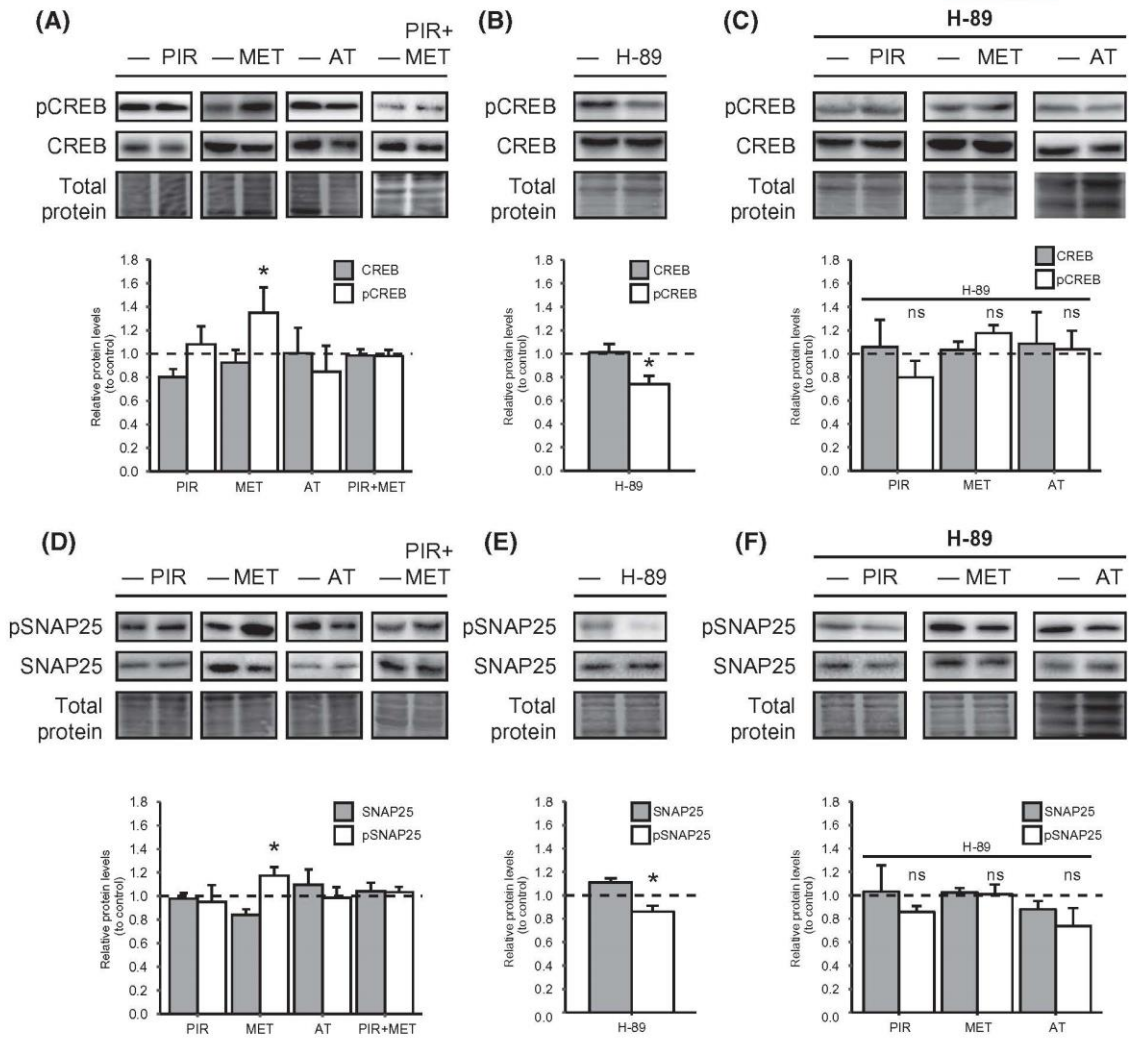

FIGURE 6 M_2 inhibits the PKA-phosphorylation of CREB and SNAP-25. Western blot analysis and data quantification of the protein levels and phosphorylation of (A-C) CREB and (D-F) SNAP-25 in the diaphragm muscle after M_1-inhibition (PIR), M_2-inhibition (MET) and M_1/M_2-inhibition by atropine (AT) and by a mixture of PIR and MET (PIR+MET; PIR, 10 μM + MET, 1 μM) with and without PKA inhibition with H-89. All data are expressed as fold change vs basal condition values: mean ± SEM. *$P < .05$; ns $P > .05$; n = 3; >5 repeats per n. Abbreviations: PIR, pirenzepine; MET, methoctramine; AT, atropine; CREB, cAMP response element binding; pCREB, Ser^{133} phosphorylated cAMP response element binding; pSNAP-25, Thr^{138}-phosphorylated synaptosomal-associated protein 25; SNAP-25, synaptosomal-associated protein 25

conclusion, Cβ is present in the three cell components of the neuromuscular synapse.

Co-immunoprecipitation of RIβ in the total lysate rescued both Cα and Cβ in the diaphragm at basal conditions, indicating that RIβ interacts with the two C subtypes (Figure 5D). Additionally, M_2 inhibition with MET decreased the ability of RIβ antibody to rescue Cα and Cβ, indicating a weaker association between RIβ and Cα and Cβ. This is in concordance with M_2 being a $G\alpha_{i/0}$-coupled GPCR, whose inhibition increases cAMP and results in the disassembling of the PKA R:C holoenzyme. Because RIβ is enriched at the synaptic region of the diaphragm, neuronally expressed, and its interaction with C subunits is modulated by M_2, RIβ is one potential mediator of M_2 signaling to modulate the PKA phosphorylation of SNAP-25 Thr^{138} (see next section) at nerve terminals of the NMJ.

3.5 | Muscarinic modulation of CREB and SNAP-25 phosphorylation by PKA

To complete the analysis of the mAChR-PKA coupling, we evaluated PKA activity after each mAChR subtype inhibition by determining the phosphorylation of the PKA substrates CREB and SNAP-25 (Figure 6A-F). CREB serine 133 (Ser^{133}) is a well-known PKA target that regulates gene

transcription. SNAP-25 is a SNARE component that is crucial for neurotransmission and it is phosphorylated by PKA on threonine 138 (Thr^{138}).

Figure 6A shows that M_1 inhibition does not affect CREB protein level and phosphorylation (pCREB: 1.1 ± 0.2; CREB: 0.8 ± 0.1). On the contrary, M_2 inhibition increased the phosphorylation of CREB (1.4 ± 0.2) without altering its protein levels (0.9 ± 0.1). This result indicates that M_2 inhibits CREB phosphorylation, probably by reducing PKA phosphorylating activity. Additionally, the M_1/M_2 muscarinic inhibition did not affect CREB phosphorylation (pCREB: 0.8 ± 0.2; CREB: 1.0 ± 0.2) further indicating the need of the M_1. Moreover, to ensure that muscarinic action on CREB is conveyed through PKA, we studied the effect of muscarinic inhibitors after PKA blockade with H-89. H-89 is a cell-permeable, potent, and reversible ATP-competitive inhibitor of PKA (Ki = 48 nM). As expected, PKA downregulation with H-89 reduced phospho-CREB level (Figure 6B; pCREB: 0.7 ± 0.1; CREB: 1.0 ± 0.2). Figure 6C shows the previous incubation with H-89 abolishes the MET effect on pCREB (pCREB: 1.2 ± 0.2; CREB: 1.0 ± 0.1). This demonstrates that M_2 inhibition of pCREB requires PKA activity. Moreover, after PKA blockade, PIR continued without affecting pCREB (pCREB: 0.8 ± 0.1; CREB: 1.1 ± 0.2) and the same for AT (pCREB: 1.0 ± 0.2; CREB: 1.1 ± 0.3), which indicates that the absence of M_1 action on CREB is not modified by PKA blockade.

Next, we measured the influence of muscarinic signaling on SNAP-25 Thr^{138}, a protein involved in the NMJ neurotransmitter release. Similarly to CREB, Figure 6D shows that M_1 inhibition does not affect pSNAP-25 Thr^{138} phosphorylation (pSNAP-25:1.0 ± 0.1; SNAP-25:1.0 ± 0.04). However, M_2 inhibition induced an increase in phospho-SNAP-25 Thr^{138} (1.2 ± 0.1) without altering its protein levels (0.8 ± 0.05). Additionally, M_1/M_2 inhibition did not affect SNAP-25 phosphorylation (pSNAP-25:1.0 ± 0.1; SNAP-25:1.1 ± 0.1). We studied the effect of H-89 on SNAP-25 Thr^{138} phosphorylation and how this inhibitor influenced muscarinic signaling. Figure 6E shows that H-89 reduces SNAP-25 phosphorylation without affecting its protein level (pSNAP-25:0.75 ± 0.1; SNAP-25:1.1 ± 0.03). Finally, Figure 6F shows also that the MET effect on pSNAP-25 can be abolished by a previous incubation with H-89, demonstrating that M_2 inhibition of pSNAP-25 requires PKA activity (pSNAP-25:1.1 ± 0.7; SNAP-25:1.0 ± 0.04).

4 | DISCUSSION

Since long time ago, PKA has been implicated in synaptic plasticity, enhancing the probability of release in the nerve terminal[8,24,71] as well as controlling the postsynaptic response.[72-74] This kinase promotes ACh release at the NMJ, remarkably via the phosphorylation of the release machinery and its regulatory components (reviewed in Leenders and Sheng, 2005). At the NMJ, the M_2 mAChR subtype inhibits PKA through the reduction of cAMP levels, a mechanism which decreases ACh release.[1,7] However, the dynamics of PKA at the NMJ upon activation remain unknown. On the other hand, the M_1 subtype couples PKC isoforms to potentiate ACh release[1,7] and PKA and PKC are also interconnected in regulating neurotransmitter release at the NMJ.[16] In the present study, we focused on the role of mAChR regulating PKA, and we found how M_2 pathway regulates PKA subunit levels, translocation and interaction in the rat diaphragm to phosphorylate representative transmitter release targets, like SNAP-25, and the PKA-dependent CREB. Moreover, we demonstrate a crosstalk between M_1 and M_2 inhibition at the molecular level that would impact in the functionality of the NMJ. Figure 7 provides a summary of the main results.

4.1 | M_1 and M_2 mutual influence

M_1 and M_2 muscarinic signaling pathways converge to antagonistically regulate ACh release at the NMJ: M_1 increases whereas M_2 decreases ACh release.[7] This muscarinic signaling is present both in basal conditions and under synaptic activity, caused by the constitutive G protein-coupled receptors activity,[75] the spontaneous ACh release,[76,77] and the evoked ACh release. Overall, M_2 signaling predominates over M_1, evidenced by M_1/M_2 inhibition increasing the quantal content similar to M_2 inhibition alone.[1,4] Here, we found that M_2 decreases the protein levels of M_1, providing a molecular explanation to previous findings of M_2 overcoming functionaly M_1. The downregulation of M_1 protein levels by M_2 might be caused by internalization and degradation[78] or by a decrease in PKA/pCREB-induced synthesis.[79] On the other hand, the downregulation of M_1 protein levels (and activity) by M_2 and not M_2 by M_1 might be due to different pathways of internalization and degradation. In particular, M_1 mAChR is internalized in a β-arrestin/dynamin-dependent manner, whereas sequestration of M_2 is largely independent of these proteins.[80,81] Interestingly, we found that M_2 needs M_1 activity to reduce the own M_1 levels, because the effect does not happen when both receptors are blocked. This receptor downregulation could be linked to PKC, as downstream kinase of M_1 mAChR which promotes GPCR kinase (GRK) activity and β-arrestin/dynamin-dependent internalization.[82,83] In fact, we found that all the M_2-induced changes in PKA subunits expression and translocation need nonblocked M_1 receptors, favoring the idea of a direct influence between M_1 and M_2. Furthermore, the results showing that while M_2 inhibits activity (and protein levels) of M_1, and that M_1 has no direct influence on M_2 could also be explained by a stronger tonic activation of M_2 than M_1 receptors. The muscarinic signaling observed in this study could be caused by

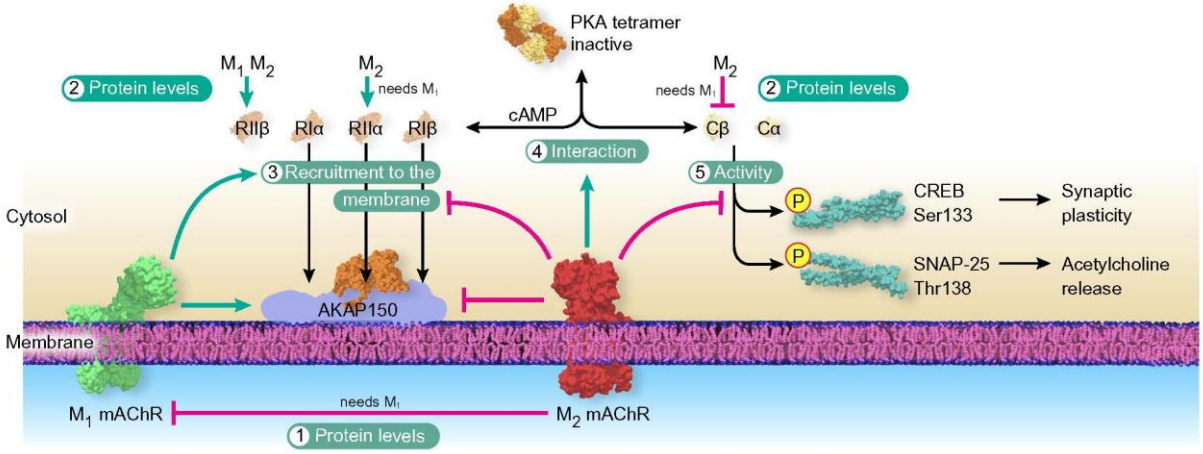

FIGURE 7 Summary of the main findings. Model of the muscarinic-PKA regulation resulted from this study. The protein kinase A (PKA) is a ubiquitous enzyme involved in neurotransmission and synaptic plasticity. The muscarinic receptor subtype M_2 is the major pathway as it reduces M_1 protein levels (1). Additionally, M_2 inhibits PKA activity by (2) downregulating the catalytic Cβ subunit, upregulating the regulatory RIIα/β and (3) liberating RIβ and RIIα to the cytosol, which promotes (4) the interaction between the synaptic-enriched RIβ and Cα/β subunits and reduces (5) CREB and SNAP-25 phosphorylation. On the other hand, M_1 signaling (2) upregulates RIIβ but (3) recruits RIα, RIβ, and RIIα to the membrane, opposed regulations which end up not affecting PKA substrates. M_1 and M_2 signaling on R subunit translocation seems similarly orchestrated through the anchoring protein AKAP150. The M2 modulations which need an active M_1 receptor are marked as "needs M_1" [Color figure can be viewed at wileyonlinelibrary.com]

the constitutive G protein-coupled receptors activity[75] or by the spontaneous ACh release.[76,77] In line with this, radiolabeling assays show that ACh has higher affinity for the pons, medulla, and heart atria, which are tissues rich in M_2 muscarinic receptors.[84,85] Additionally, functional assays at the adult neuromuscular junction indicate that M_1/M_2 signaling is sensitive to ACh concentrations at the murine NMJ and that M_2 inhibition of neurotransmitter release overcomes the signaling of M_1 under normal conditions.[1,2,7,64] Our results also showed that the mixture PIR+MET fully mimicked the effect of atropine in all the conditions tested. The mix of inhibitors would yield results with less participation of other mAChR subtypes. However, it has been determined that the M_3 and especially M_4 subtypes only participate in the development of newborn NMJ,[63-65] and that M_1 and M_2 subtypes orchestrate the release at the adult NMJ.[2,63,66] The present results are a complementary evidence of this. Slutsky et al[4] also showed that the mixture of PIR+MET fully mimicked the effect of atropine in the frog NMJ. Also, Brazhnik et al[67] compared the effect of the pan-muscarinic inhibitor scopolamine (a drug almost identical to atropine except for an epoxide group with the effect of a PIR+MET mixture and showed that effects of scopolamine were mimicked by the mixture.

4.2 | Muscarinic modulation of PKA catalytic and regulatory subunits protein level

Besides cAMP availability, PKA activity is also regulated by its concentration through transcriptional or posttranscriptional changes in the synthesis or degradation of its subunits.[47,72,86] In this work, we found that both M_1-PKC and M_2-PKA pathways regulate the protein levels of specific PKA subunits. The unexpected role of M_1-PKC pathway could be related to the few studies finding that both PKC and PKA activities can modify the expression of PKA subunits (particularly Cα and RIIβ) in cultured cells.[47,87] Despite being generally considered G_q specific, M_1 has also been linked to PKA stimulation via G_s alpha subunit in cell cultures.[88,89] Additionally, another cross-link between M_1 and M_2 pathways could be through a calcium-induced reduction of cAMP.[90,91] Regarding M_1 upregulation of RIIβ and M_2 downregulation of Cβ and upregulation of RIIα/β, both receptors could apparently be associated to a decrease in PKA catalytic activity because increased turnover of C subunits or elevated levels of R subunits results in reduced catalytic activity.[48] The downregulation of Cβ might affect the synaptic machinery, because Cβ is highly expressed in the nervous system[92,93] and we found it abundant in the synaptic region of the diaphragm and present at the nerve terminal of the NMJ (and also in the other two synaptic cell components) like SNAP-25.[55] Interestingly, we also found this M_2 effect over the Cβ subunit when the phrenic nerve is stimulated. Moreover, phrenic nerve stimulation by itself increases Cβ levels indicating that M_2 mAChR signaling opposes to the Cβ-enhancing action of nerve stimulation. Both M_1 and M_2 increase RIIβ levels, which is a link between M_1 and M_2 pathways that could inhibit PKA activity and allowing M_1 to indirectly influence

M_2 signaling. Interestingly, the downregulation of RIIβ by AT or the mixture PIR+MET seems to be less that that observed when M1 and M2 are inhibited separately. This inability to further inhibit the levels of RIIβ with AT or PIR+MET might be explained by mechanisms other than muscarinic receptors which could be buffering and protecting the synapse from an excessive decrease of the subunit. At the NMJ, muscarinic and neurotrophic signaling pathways confluent downstream in PKA and PKC to regulate ACh release indicating the possible crosslinking of these signals.[94] We know that exogenous BDNF increases RIIβ (unpublished result) suggesting that the BDNF/TrkB pathway could be preventing RIIβ levels from falling excessively. In addition, M_2 blockade also decreases the regulatory subunit RIIα. This indicates that the two isoforms of RII are involved in the muscarinic downregulation of the PKA activity. In addition, the regulation of Cβ by M_2 also occurs in presence of cholinergic input from phrenic nerve, demonstrating a general mechanism of regulation, both tonicaly and in physiological condition. In fact, it has been described that constitutive activity of neurotransmitter GPCRs may provide a tonic support for basal neuronal activity (reviewed by 75). Here, we show that tonic and activated muscarinic signaling largely modulates proteins responsible of regulating synaptic transmission and plasticity, suggesting that the signaling pathway elicited by muscarinic receptors works in transmission of NMJ. Interestingly, both Cβ and RIIα changes by M_2 need M_1 activity, indicating the cooperation of both mAChR pathways to control PKA activity. In summary, M_1 and M_2 receptors regulate the protein level of several PKA subunits. This presumably reduces PKA activity as it has been described that increased levels of R subunits reduce catalytic activity.[48]

4.3 | PKA subunits membrane-cytosol translocation is modulated by mAChR

The PKA distribution between the membrane and cytosolic compartments has implications in their kinase activity and specificity.[24,41] In our model, C subunits are mainly located in the cytosol and the presence of R subunits in the cytosol is required for the negative regulation of the PKA catalytic activity. We detected that PKA Cα, Cβ and RIα, RIβ, and RIIβ subunits are mainly associated to the cytosol fraction (~75% cytosol/total) and only RIIα had less presence in the cytosol (60%). This is consistent with reports that PKA RI type is diffused in the cytoplasm of cells, whereas RII type is usually associated to membrane compartments[33,35,41,46] due to their higher affinity to PKA-anchoring proteins.[43] PKA activity can be regulated by cytosol/membrane translocation of both C[40] and R subunits.[41,42] In the adult rat diaphragm, we found that mAChR signaling regulates the translocation of PKA R subunits rather than C subunits. Our results indicate that M_1 receptors constitutively recruit RIα, RIβ, and RIIα to the membrane, whereas M_2 receptors liberate RIβ and RIIα to the cytosol. Also, the absence of effect when both M_1 and M_2 are blocked with AT could be because their opposed actions cancel each other out or, alternatively, because one subtype is necessary for the other. We speculate that the coexistence of both mAChR subtypes may balance the presence of RIβ and RIIα in the cytosol to finely control PKA C activity. RI subunits are known to associate with membrane fractions when they are not associated with C subunits.[41] In this regard, a similar mechanism was proposed by Stefan et al,[42] where a GPCR-mediated cAMP elevation promotes dissociation of the PKA heterotetramer and recruitment of R subunits to $G\alpha_{i/0}$ proteins at the membrane. Thus, the recruitment to the membrane of RIβ and RIIα produced by the M_2 inhibitor methoctramine could be linked with the increase in cAMP produced by the inhibitor. Also, the eventual prevalence of M_2 downstream signaling in basal conditions at the NMJ[1] as well as the M_2-induced decrease of M_1 would promote the release and presence of R subunits in the cytosol. The close association of the regulatory and catalytic subunits prevents the phosphorylating activity of the catalytic ones (Reviewed in 24), and we show that M_2 signaling tonically maintains RIβ strongly associated with Cβ and Cα at the synaptic areas of the skeletal muscle. However, further experiments about the interaction between subunits will be needed to better understand the mechanism of inhibition of the C subunit by the R ones.

Therefore, M_1 receptor constitutively maintains PKA RIα, RIβ, and RIIα in the membrane fraction, whereas the tonic effect of M_2 mAChR might inhibit the action of M_1, enhancing the release of PKA RIβ and RIIα to the cytosol. This mAChR-modulated balance of the membrane-cytosol position of these subunits involves a mechanism that include the anchoring protein AKAP150 and may be relevant in PKA activity regulation and specificity. The mechanisms that regulate AKAP150 expression and degradation remain unclear. Here, we demonstrate that M_1 mAChR, known to potentiate ACh release, upregulates AKAP150. Contrarily, we found that M_2 mAChR downregulates AKAP150. Changes in AKAP150 expression have been determined in other systems.[95-97] In particular, impaired Ca^{2+} cycling in a heart failure model[97] or induced by PIR (our results) has been linked to AKAP150 downregulation. On the other hand, exercise training in rats[95] and ACh release upregulation by MET (our results) increases AKAP150 indicating that M_1 can increase the association PKA R subunits to the membrane, thus increasing the cytosolic activity of C subunits. Targeting of PKA to specific sites within the cell is largely achieved by AKAPs.[43,98] Also, R translocation to

the membrane aside from AKAP150 could also been related to $G\alpha_{i/0}$ association.[42] Additionally, AKAP150 has been shown to be also an anchoring protein for PKC[99-101] and it could be possible that the M_1 upregulation of AKAP150 affects PKC contributing thus to the functional and molecular relation between PKC and PKA to regulate neurotransmission at the NMJ. Results using kinase blockers indicate that the PKC isoform epsilon (PKCε), the PKC isoform beta I (PKCβI) and PKA promote AKAP150 protein levels and their action is involved in the M_1-upregulation and the M_2-downregulation of AKAP150 (results not shown). It is stimulating to think that multiprotein complexes orchestrated by AKAPs create presynaptic membrane sites where in signaling pathways converge and are regulated to optimize the functionality of the NMJ. Thus, at the NMJ, a cross-linking between M_1 and M_2 could include PKA and PKC actions through direct attach to AKAP150 in the membrane close to the PKA and PKC targets to modulate neurotransmission release (see Figure 8A). This would be in concordance with the functional connection of PKA and PKC to regulate neurotransmitter release at the NMJ.[16]

4.4 | M_2 inhibits PKA activity on CREB and SNAP-25 phosphorylation

PKA phosphorylates many molecular targets related with immediate transmitter release or with long-lasting regulation of neurotransmission. Some PKA targets directly involved in transmitter release are N-ethylmaleimide-sensitive factor attachment protein alpha (α-SNAP),[102] the cysteine string protein (CSP),[103] synapsin I,[104] snapin,[105] syntaphilin,[106] rabphilin,[107] Rab3 interacting protein 1α (RIM1α),[108] and SNAP-25.[17,18] SNAP-25, together with synaptobrevin and syntaxin are the three SNARE proteins of the core fusion vesicle complex, which is involved in vesicle docking, priming, and triggering fast exocytosis.[109,110] It has been proposed that PKA phosphorylation of SNAP-25 at Thr[138]

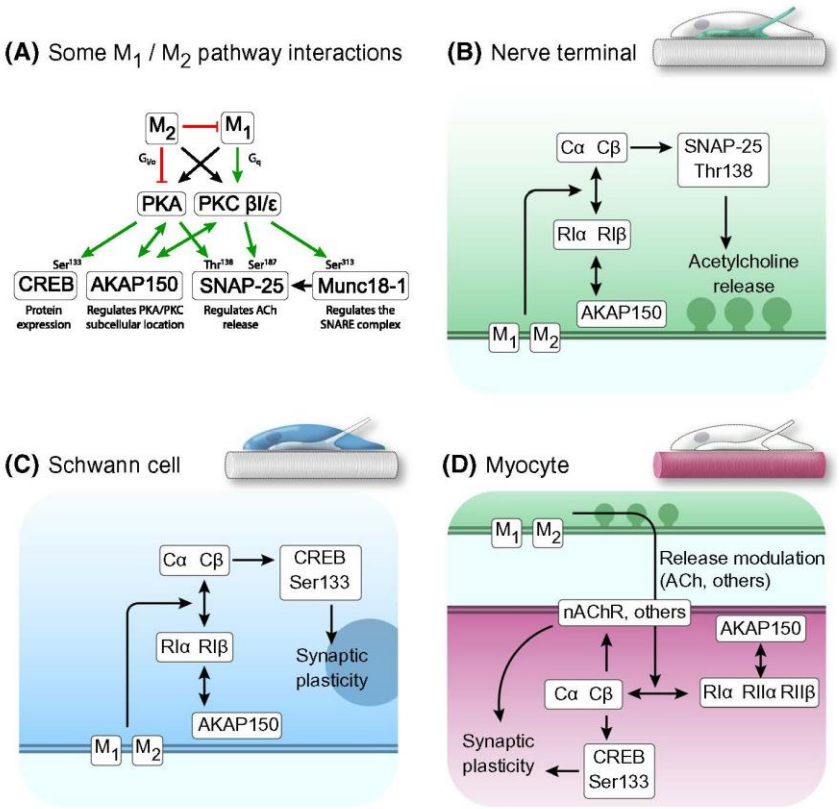

FIGURE 8 Hypothesis of muscarinic M_1/M_2 interaction (A) and cell-specific action at the neuromuscular junction (B-D). (A) Although the classic vision of M_1 and M_2 muscarinic signaling divides their signaling in G_q/PKC and $G_{i/0}$/PKA, respectively, recent findings show these GPCR share multiple downstream effectors to finely regulate ACh release in an opposed manner at the NMJ. (B-D) Cell-specific models of the muscarinic-PKA regulation in (B) the nerve terminal, (C) the Schwann cell, and (D) the postsynaptic myocyte notated based on the current knowledge. AKAP150, A kinase anchor protein 150; CREB, Ser^{133}-phosphorylated cAMP response element binding; Cα/β, protein kinase A catalytic subunit α/β; M_1, muscarinic receptor subtype 1; M_2, muscarinic receptor subtype 2; RIα/RIβ/RIIα/RIIβ, protein kinase A regulatory subunit Iα/Iβ/IIα/IIβ; SNAP-25, synaptosomal-associated protein 25 [Color figure can be viewed at wileyonlinelibrary.com]

controls the size of the releasable vesicle pools, whereas the novel PKCε phosphorylation of SNAP-25 at Ser[187] is involved in regulating refilling after the pools have been emptied.[9,55,111] Here, we found that M_1 inhibition does not affect SNAP-25 Thr[138] phosphorylation but M_2 inhibition induced an important increase which can be abolished by a previous incubation with H-89, demonstrating that M_2 inhibition of pSNAP-25 involves the modulation of the PKA activity. We previously determined that M1/M2 inhibition by atropine increases the quantal release.[15] However, the block of both M1 and M2 receptors with AT and the mixture PIR+MET did not affect SNAP-25 Thr[138] and CREB Ser[133] phosphorylation, indicating that M2 needs M1 operativity. This is in line with biochemical studies showing that PKA-dependent phosphorylation of SNAP-25 does not regulate ternary SNARE complex assembly and that Thr[138] is not an essential residue for complex formation or its stability.[17] The absence of effect on SNAP-25 Thr[138] indicates that atropine effect on quantal ACh release should be understood by other PKA and PKC substrates, including SNAP-25 Ser[187] (PKC),[18,112] Munc18-1,[9,58] synaptotagmin,[113] N-ethylmaleimide-sensitive factor (NSF),[114-116] N-type calcium channels, voltage-gated Na^+ channels,[122-124] and the myristoylated alanine-rich C-kinase substrate (MARCKS).[125]

CREB is a long-lasting master regulator of gene expression, whose activity has been linked to synaptic plasticity at the NMJ as well as in the central nervous system.[20] CREB is phosphorylated by various kinases, for instance, cAMP/PKA, ERK1/2, and PI3K/Akt (reviewed by 9,117) and we selectively analyzed the PKA/CREB signaling pathway. Similarly to SNAP-25 phosphorylation, M_1 inhibition does not affect CREB phosphorylation but M_2 inhibition increased it indicating that M_2 normally inhibits PKA phosphorylating activity on CREB. The M_2 inhibition effect on pCREB can be markedly reduced by a previous incubation with H-89, indicating that M_2 inhibition of pCREB requires PKA activity. Our ex vivo approach maintains the architecture of synapses as the in vivo conditions and allows to study an accurate version of the behavior and function of the neuromuscular system. However, PKA is widely expressed and some mechanisms described here could occur in different cell types. For example, CREB expression and phosphorylation at the skeletal muscle occurs in nuclei of myocytes and is differentially activated in synaptic and extrasynaptic regions of fast- and slow-twitch muscles.[21]

The mAChR M_1 and M_2 subtypes are present at the nerve terminal but also at the perisynaptic glial cell,[6] suggesting that mAChR signaling could be initiated in both cells (Figure 8B,C). However, although it has been demonstrated the presence of mAChR in rat cultured skeletal muscle membrane and developing muscle fibers, the innervated adult skeletal myocytes do not express mAChRs.[66,118,119,121] Therefore, in case that some of the mAChR effects on PKA subunits and/or the phosphorylation of its targets might occur in the postsynaptic cell, it would be an evidence of the communication between cells through the signaling and indicate the complexity of the mAChR regulation (Figure 8D). Stabilization of the nAChR at the postsynaptic membrane is related with PKA activity[112-114,120,122] and in particular by the RIα subunit[36,115,116] and we could think that this mechanism might be anterogradely regulated by mAChR signaling.

The block of both M_1 and M_2 receptors with AT and the mixture PIR+MET did not affect SNAP-25 and CREB phosphorylation indicating that M_2 needs M_1 operativity. M_1 modulates also the translocation to the membrane of several R subunits and the protein level of RIIβ though without consequences in CREB and SNAP-25 phosphorylation. This suggests that the M_2 inhibition of Cβ and translocation of R subunits to the cytosol could be responsible for the reduced phosphorylation of SNAP-25 and CREB although needs M_1 active.

5 | CONCLUDING REMARKS

It is known that the activation of M_2 subtype mAChR begins a Gi protein-coupled downstream signal that inhibits adenylyl cyclase, cAMP levels and PKA activity with the subsequent decrease in ACh release at the NMJ. In the present work, we determined the dynamics of PKA subunits at the NMJ providing a molecular mechanism of the PKA holoenzyme formation and inactivation upon constitutive and activated muscarinic signaling that could be general to other synapses and cellular models. The M_2 action reduces the Cβ protein level, increases RIIα and RIIβ, and translocates RIβ and RIIα to the cytosol with the involvement of the anchoring protein AKAP150. This coincides with a decrease in the phosphorylation level of the master regulator of gene expression CREB and SNAP-25 that are PKA targets and with a decrease in the neurotransmission.[1] Some of the M_2-induced changes need an active M_1 receptor (reduction of the Cβ and increase of the RIIα), while some other M_2-induced change can be additionally produced also by M_1 receptor action (the increase of the RIIβ protein and their release to the cytosol). On the other hand, M_1 receptor seems to produce several changes that would be interpreted as opposed to M_2 by holding RIα, RIβ, and RIIα in the membrane fraction. Reciprocally, M_2 reduces M_1 protein level. Altogether, these results reveal the complexity of PKA expression and regulation by muscarinic signaling and points to the operation of a balance M_1/M_2 in the regulation of the PKA activation that could be orchestrated by AKAP150. At the nerve terminal of the NMJ, the tonic action of the M_2 receptor to decrease ACh release would stabilize the holoenzymes formed by Cα and Cβ with at least RIβ. This coincides with the final reduction in the phosphorylation level of the essential exocytotic protein SNAP-25.

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COMPETING INTERESTS

The authors declare no competing financial interests.

ETHICS STATEMENT

The animals were cared for in accordance with the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All the procedures realized were revised and authorized by the Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 0289).

AUTHOR CONTRIBUTIONS

N. Garcia, J.M. Tomàs, and M.A. Lanuza designed research; V. Cilleros-Mañé and L. Just-Borràs performed research; V. Cilleros-Mañé, L. Just-Borràs, M. Tomàs, N. Garcia, J.M. Tomàs, and M.A. Lanuza analyzed data. V. Cilleros-Mañé, L. Just-Borràs, J.M. Tomàs, and M.A. Lanuza wrote the paper. All authors read and approved the final version.

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Publication 2

M_1 and M_2 mAChRs activate PDK1 and regulate PKCβI and ε and the exocytotic apparatus at the NMJ

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RESEARCH ARTICLE

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M_1 and M_2 mAChRs activate PDK1 and regulate PKC βI and ε and the exocytotic apparatus at the NMJ

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Abstract

Neuromuscular junctions (NMJ) regulate cholinergic exocytosis through the M_1 and M_2 muscarinic acetylcholine autoreceptors (mAChR), involving the crosstalk between receptors and downstream pathways. Protein kinase C (PKC) regulates neurotransmission but how it associates with the mAChRs remains unknown. Here, we investigate whether mAChRs recruit the classical PKCβI and the novel PKCε isoforms and modulate their priming by PDK1, translocation and activity on neurosecretion targets. We show that each M_1 and M_2 mAChR activates the master kinase PDK1 and promotes a particular priming of the presynaptic PKCβI and ε isoforms. M_1 recruits both primed-PKCs to the membrane and promotes Munc18-1, SNAP-25, and MARCKS phosphorylation. In contrast, M_2 downregulates PKCε through a PKA-dependent pathway, which inhibits Munc18-1 synthesis and PKC phosphorylation. In summary, our results discover a co-dependent balance between muscarinic autoreceptors which orchestrates the presynaptic PKC and their action on ACh release SNARE-SM mechanism. Altogether, this molecular signaling explains previous functional studies at the NMJ and guide toward potential therapeutic targets.

KEYWORDS
muscarinic receptors, neuromuscular junction, PDK1, PKC, SNAP-25

Abbreviations: ACh, acetylcholine; Atr, atropine; Ca^{2+}, calcium; cPKC, classic PKC isoform; Dk, donkey; M_1, muscarinic acetylcholine receptor 1 subtype; M_2, muscarinic acetylcholine receptor 2 subtype; mAChR, muscarinic acetylcholine receptor; MARCKS, myristoylated alanine-rich C-kinase substrate; Met, methoctramine; Ms, mouse; Munc18-1, mammalian homologue of UNC-18; NMJ, neuromuscular junction; nPKC, novel PKC isoform; Pir, pirenzepine; PKC, protein kinase C; PKCβI, protein kinase C isoform Bi; PKCε, protein kinase C isoform ε; RACK1, receptor for activated C-kinase 1; Rb, rabbit; SNAP-25, synaptosome-associated protein 25; $βIV_{5-3}$, PKCβI inhibitor peptide; $εV_{1-2}$, PKCε inhibitor peptide.

N. Garcia, J. M. Tomàs, and M. A. Lanuza are equally contributed to this work.

1 | INTRODUCTION

Neuromuscular junctions (NMJ) express muscarinic acetylcholine receptors (mAChR) to sense how much acetylcholine is released and tune exocytosis in feedback. This coordination is achieved through the potentiating and inhibitory actions of M_1 and M_2 mAChR subtypes, respectively.[1] In general, the M_1 mAChR is classically associated to $G\alpha_q$ proteins and protein kinase C (PKC), whereas the M_2 mAChR is linked to $G\alpha_i$ proteins and protein kinase A (PKA) inhibition. However, this essential signaling is highly complex because it involves multiple downstream transduction pathways and the crosstalk between receptor subtypes. An example of this complexity at the NMJ is that the M_2 muscarinic receptor needs the association to M_1 to regulate the neuromuscular PKA molecular dynamics.[2] Also, the selective inhibition of both mAChR subtypes induce PKC action on NMJ neurotransmission[1] in which PKC plays an essential role.[3,4] At the adult NMJ, PKC coupling to ACh release requires a stimulus like calcium, presynaptic stimulation or the modulation of mAChR.[1,3,5] However, the molecular PKC signaling coupled to the ACh release associated to these signals, including the mAChR regulation, remains to be elucidated.

Previously to its activation, PKC undergoes a process of maturation (priming) to become competent to respond to second messengers.[6,7] PKC maturation involves three phosphorylation steps: the first is mediated by the phosphoinositide-dependent kinase 1 (PDK1) in the activation loop of the catalytic domain and the second and third are PKC autophosphorylations in the turn and hydrophobic motifs of the carboxy-terminal region.[8,9] Once matured, PKC stays in the cytosol in an inactive conformation ready to be activated.[9-11] Although PKC maturation was initially seen as constitutive, further studies found several stimuli which can induce it.[12-15] Finally, PKC activation requires its recruitment to the membrane, which is driven by binding to calcium, diacylglycerol and phosphatidylserine for classical PKC isoforms (cPKCs) and diacylglycerol and phosphatidylserine for novel PKC isoforms (nPKCs).[16] To end their signal, active PKCs are prone to be ubiquitinated and undergo activation-induced degradation.[17-20]

PKC phosphorylates many targets, which participate in general intracellular processes as well as neurotransmitter release. However, little is known about the role of each PKC isoform. Determining how extracellular receptors recruit specific PKC isoforms is crucial to predict how cells respond to extracellular signals. The cell components of the NMJ express different PKC isoforms, which likely help to finely regulate ACh release.[21-23] Of particular interest are PKCβI and PKCε, which are exclusive of the presynaptic nerve terminal and essential for ACh release.[22,24] For example, PKC phosphorylates the Ser^{306} and Ser^{313} of the accessory SNARE protein Munc18-1 (mammalian homologue of UNC-18), an essential, neuron-specific protein involved in neurotransmitter release[25-28] to prime vesicle fusion and increase the pool available for release.[29,30] At the NMJ, PKCβI and PKCε isoforms coordinately regulate Munc18-1 activity-dependent phosphorylation.[31] Another PKC substrate is SNAP-25 (synaptosome-associated protein 25), a component of the SNARE core complex. PKC phosphorylates SNAP-25 on Ser^{187}, a critical residue for calcium-triggered exocytosis.[28,32-36] This phosphorylation occurs after synaptic activity and high intracellular calcium and promotes vesicle pool refilling.[4,36,37] At the NMJ, PKCε regulates its activity-dependent phosphorylation.[38] Another example is MARCKS (myristoylated alanine-rich C-kinase substrate), a widely distributed PKC substrate which rearranges actin in the cytoskeleton in response to extracellular signals. Its phosphorylation is a marker of PKC activation in vivo[39] and it is also implicated in cholinergic neurosecretion and membrane trafficking.[40] At the NMJ, MARCKS phosphorylation has been linked with PKCε activity.[23]

Although PKC plays an essential role at the neurotransmission at the NMJ,[1] it is unknown the molecular PKC signaling coupled to the ACh release associated to its inducer signals, including the mAChR regulation. In the present work, we investigate whether M_1 and M_2 mAChRs regulate the PDK1-induced priming and recruit the classical PKCβI and the novel PKCε isoforms and modulate their maturation, membrane translocation and the phosphorylation of Munc18-1, MARCKS, and SNAP-25, crucial targets for neurosecretion. To analyze it, we studied the effect of muscarinic blockade on PDK1, PKCβI, and PKCε isoforms and their targets. Our results show a novel link between M_1 and M_2 signaling and the master kinase PDK1 and highlight the relevance of the balance between the presynaptic muscarinic autoreceptors M_1 and M_2 to influence a pool of PKC isoforms, which finely tuned the ACh release SNARE-SM mechanism.

2 | MATERIALS AND METHODS

2.1 | Animal welfare

The animals were cared for in accordance with the European Community Council Directive guidelines for the humane treatment of laboratory animals. Adult Sprague-Dawley rats (40-80 days; Criffa, Barcelona, Spain) were euthanized for tissue harvest and analysis. Animals were randomly assigned to the different treatments and at least three animals ($n \geq 3$) were used as biological replicates for every experiment detailed below. All animal work was approved by the Ethics Comitee of Animal Experimentation of the Universitat Rovira i Virgili.

2.2 | Chemicals

Muscarinic inhibition. Pirenzepine dihydrochloride (Tocris): 10 mM stock and used at 10 μM. Methoctramine tetrahydrochloride (Sigma): 1 mM stock and used at 1 μM. Atropine (Sigma): 200 μM stock and used at 2 μM.

PKC inhibition. The activity of PKC isoforms was blocked with PKC-derived peptides which compete for the receptor for activated C-kinase 1 (RACK1) binding and disrupt the cellular targeting of PKC isoforms. The PKCβI inhibitor βIV_{5-3} peptide[41,42] was kindly provided by Dr Mochly-Rosen from Stanford University and the PKCε inhibitor εV_{1-2} peptide[43] from MERCK. Both peptides are <40 amino acids (βIV_{5-3}, CKLFIMN; εV_{1-2}, EAVSLKPT). Briefly, blocking PKCβI with βIV_{5-3} did not affect PKCβII[44] and blocking PKCε with εV_{1-2} did not affect the novel PKCδ or classical PKC isoforms.[43,45,46] Furthermore, εV_{1-2} peptide has been validated with PKCε knockout mice.[47,48] Also, multiple sequence alignment reveals that βIV_{5-3} peptide shares 100% identity with PKCβI (Uniprot ID: P68403-1) and 0% identity with PKCε (Uniprot ID: P09216). Additionally, εV_{1-2} peptide shares 0% identity with PKCβI and 100% identity with PKCε. Working concentrations were optimized to 10 μM for βIV_{5-3}[22] and 100 μM for εV_{1-2}.[23] The difference in concentration was due to βIV_{5-3} peptide being connected to a deliverer peptide to enhance cell penetration.

PDK1 inhibition. PDK1 activity was blocked with GSK2334470, from MERCK. This highly specific inhibitor, only inhibited PDK1 activity without affecting 93 other kinases screened, including PKC and PKA.[49] GSK2334470 was made as 5 mM stock in DMSO and used at 2 μM on excised diaphragm muscles.

PKA inhibition. PKA activity was blocked with N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89, Calbiochem). H89 was made as 5 mM stock and used at 5 μM.

All chemicals were diluted in Ringer as specified and both control and drug-containing solutions contained dimethyl sulfoxide as vehicle at a final concentration of 0.1% (v/v).

2.3 | Treatments

The treatments were performed ex-vivo on excised diaphragm muscles. The experimental design was pairwise: one hemidiaphragm underwent the treatment while the other served as its paired control. In single-inhibitor treatments, the treated preparation was incubated for 60 minutes in Ringer solution containing the inhibitor. We used double-inhibitor treatments to study the implication of PKC in muscarinic signaling. In these, the treated hemidiaphragms were first preincubated for 30 minutes in Ringer solution containing a kinase inhibitor (βIV_{5-3}; εV_{1-2} or H89) and afterwards for further 30 minutes in Ringer solution containing the kinase inhibitor plus the muscarinic inhibitor indicated. The control pairs of the doubly inhibited preparations were incubated for 60 minutes in Ringer solution containing the appropriate kinase inhibitor to normalize its effects.

2.4 | Sample processing and fractionation

The diaphragm muscle was obtained from adult Sprague-Dawley rats (P40-60) immediately after euthanasia. Unconsciousness was induced by an intraperitoneal injection of tribromoethanol 2% (0.15 mL/10 g body weight) and afterwards euthanasia was performed through anaesthesic overdose. As indicated in the Directive 2010/63/EU and the Real Decreto 53/2013, death was confirmed by exsanguination. Diaphragms were excised with the phrenic nerve, taking special care to isolate the same nerve length and avoid connectivity damage.

Whole cell lysate. After treatment, muscles were immediately frozen in liquid nitrogen. Homogenization was performed with an overhead stirrer (VWR International, Clarksburg, MD) in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; NP-40 1%, Triton X-100 0.1%, and protease inhibitor cocktail 1% (Sigma, Saint Louis, MO, USA)). Insoluble materials were removed with two centrifugations at 4°C: 1000g for 10 minutes and 15000g for 20 minutes. The final supernatant contained the whole cell fraction lysate.

Membrane/cytosol fractionated lysates. For membrane-cytosol fractionation, samples were immediately homogenized without freezing to avoid membrane damage before purification. The lysis buffer did not contain detergents (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; and protease inhibitor cocktail 1%). Insoluble materials were removed by centrifugation at 1000 *g* for 15 minutes at 4°C. The resulting supernatant was further centrifuged at 130 000 g for 1 hour. The new supernatant corresponded to the cytosolic fraction while the pellet to the membrane fraction. The latter was resuspended in lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; NP-40 1%, Triton X-100 0.1%, and protease inhibitor cocktail 1%). The purity of the subcellular fractionation was determined with the cytosol-specific GAPDH and the membrane-specific Na^+/K^+-ATPase.

2.5 | Antibodies

The primary and secondary antibodies used are listed in Table 1. The anti-PKCε and anti-PKCβI antibodies were raised against their C-terminal region (human peptide), which share a low identity percentage (45%) between each

TABLE 1 Primary antibodies

Target	Immunogen	Origin	Company (ref)	Dilution
PDK1	Hu PDK1 residues 229-556	Ms mAb	Santa Cruz (sc-17765)	1/1000
pPDK1 (pSer^{241})	Hu PDK1 residues around pSer^{241}	Rb pAb	CST (3061)	1/1000
PKCβI	Hu PKCβI C-terminus	Rb pAb	Santa Cruz (sc-209)	1/1000
pPKCβI (pThr^{642})	Hu PKCβI residues 640-644	Rb pAb	Abcam (ab75657)	1/1000
PKCε	Hu PKCε C-terminus	Rb pAb	Santa Cruz (sc-214)	1/1000
pPKCε (pSer^{729})	Hu PKCε residues around pSer^{729}	Rb pAb	Santa Cruz (sc-12355)	1/1000
PKCα	Hu PKCα C-terminus	Rb pAb	Santa Cruz (sc-209)	1/1000
pPKCα (pSer^{657})	Phosphopeptide corresponding to the residues 654-663	Rb pAb	Upstate (06-822)	1/1000
Munc18-1	Hu Munc18-1 residues around Tyr^{157}	Rb mAb	CST (13414)	1/1000
pMunc18-1 (pSer^{313})	Hu Munc18-1 residues 307-319	Rb pAb	Abcam (ab138687)	1/1000
SNAP-25	Hu SNAP-25 residues around Gln^{116}	Rb mAb	CST (5309)	1/1000
pSNAP-25 (pSer^{187})	Rat SNAP-25 residues around pSer^{187}	Rb pAb	Abcam (ab169871)	1/1000
MARCKS	Hu MARCKS residues 2-66	Ms mAb	Santa Cruz (sc-100777)	1/1000
pMARCKS (pSer$^{152/156}$)	Rat MARCKS residues around pSer$^{152/156}$	Rb pAb	Sigma (07-1238)	1/1000
GAPDH	Rb GAPDH	Ms mAb	Santa Cruz (sc-32233)	1/2000
ATPase	Chicken ATPase residues 27-55	Ms mAb	DSHB (a6f)	1/2000
Secondary antibodies	Anti-Rb conjugated HRP	Dk pAb	711-035-152	1/10000
	Anti-Ms conjugated HRP	Rb pAb	A9044	1/10000
	Anti-goat conjugated Alexa fluor 568	Dk pAb	A-11057	1/500

Note: Antibodies used in this study and procedure specifications.

Abbreviations: Dk, donkey; Hu, human; mAb, monoclonal antibody; Ms, mouse; pAb, polyclonal antibody; Rb, rabbit.

other. These antibodies were validated in Hurtado et al and Simó et al.[22,31] In brief, the incubation with the εV_{1-2} peptide for 30 minutes decreases PKCε and pPKCε levels and the incubation with the βIV_{5-3} peptide decreases PKCβI and pPKCβI levels. The anti-Munc18-1 antibody epitope, the residues surrounding Tyr^{157}, is not conserved in other Munc18 isoforms, and the anti-pMunc18-1 Ser^{313} antibody was raised against a synthetic peptide corresponding to the

human Munc18-1 region 307-319, around the PKC target. The anti-SNAP-25 antibody was raised against the human peptide surrounding residues of Gln^{116}, which are not conserved in other SNAP family members (identity percentages in rat: SNAP-25 100%; SNAP-23 63%; SNAP-47 25%; SNAP-29 13%). SNAP-25 antibody showed the typical pattern of tissue expression previously known, different from SNAP-23,[50] making cross-reactivity less likely. On the other hand, phosphorylated SNAP-25 (pSNAP-25) at Ser^{187}, was detected with an antibody raised against the residues 182-192 of the protein. This sequence differs from SNAP-23 (identity percentage in rat: 73%); SNAP-47 (9%), and SNAP-29 (27%). Sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw).[51] Moreover, the phosphospecificity was proven by the absence of signal after incubation with the antigen phosphopeptide[52] and after treatment with lambda phosphatase (manufacturer's datasheet).

As a control for western blot, when primary antibodies were omitted, the membranes never revealed staining due to the secondary antibody. Pretreatment of a primary antibody with the appropriate blocking peptide (between three- and eightfold by weight) in skeletal muscle tissue prevented immunolabeling. The incubation with the specific εV_{1-2} peptide for 30 minutes decreases PKCε and pPKCε levels, whereas the incubation with the βIV_{5-3} peptide decreases PKCβI and pPKCβI levels.[31] As a control for immunohistochemistry, several muscles were incubated omitting the primary antibody, which always abolished any positive staining.

2.6 | Western blotting

Sample protein content was determined with the DC protein assay (Bio-Rad, CA, USA). Volumes containing 30 μg of protein were separated at 110 V through 8% SDS-PAGE gels (10% to detect SNAP-25) and electrotransferred to PVDF membranes (Bio-Rad, CA, USA). Blocking solutions were tris-buffered saline with Tween-20 containing 5% nonfat dry milk or 5% BSA. Primary antibodies were incubated overnight at 4°C and HRP-conjugated secondary antibodies for 1 hour. Chemiluminescence was revealed with an ECL kit (GE Healthcare Life Sciences, UK) and imagined with the ChemiDoc XRS+Imaging System (Bio-Rad, CA, USA).

The optical density of the bands was calculated with ImageJ software, always from the same immunoblot image. The values were normalized to the background value and to the total protein transferred, analyzed with SYPRO Ruby protein blot stain, (Bio-Rad, CA, USA). Foldchanges between treatment and control were always calculated from the same immunoblot image. All presented data derive from densitometry measurements made of 3-10 separate replicates, plotted against controls.

2.7 | Immunohistochemistry

The NMJ of diaphragm and *levator auris longus* (LAL) muscles were analyzed by immunohistochemistry with identical outcomes. The thinness of LAL muscles improves image quality and analysis of NMJs. Whole muscle mounts were fixed with 4% paraformaldehyde for 30 minutes. After fixation, the muscles were rinsed with PBS and incubated in 0.1 M glycine in PBS. The following incubations were performed overnight at 40°C: first, permeabilization with 1% Triton X-100 in PBS. Then, blocking of nonspecific binding with 4% BSA in PBS. Next, muscles were incubated also overnight with the appropriate primary antibody. After five washing steps, the muscles were incubated at 4°C with the appropriate secondary antibody together with α-bungarotoxin (α-BTX) conjugated with TRITC, to detect nicotinic acetylcholine receptors (nAChRs). Immunolabeled NMJs from the whole-mount muscles were viewed with a laser-scanning confocal microscope (Nikon TE2000-E). Special consideration was given to the possible contamination of one channel by another. In experiments involving negative controls, the photomultiplier tube gains and black levels were identical to those used for a labeled preparation made in parallel with the control preparations. Images were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA) and neither the contrast nor brightness were modified.

2.8 | Statistics

The ratio between the experimental and control samples was calculated densitometry measures of the same image Values are presented as mean ± standard deviation (SD). Shapiro-Wilk test was used to test sample normality. Statistical difference was determined with paired Student's *t* test or its non-parametric alternative Wilcoxon test. Multiple comparisons were corrected with the Holm-Sidak method (GraphPad Prism, San Diego, USA). The significance threshold was $*P < .05$, $**P < .01$, and $***P < .001$.

3 | RESULTS

3.1 | M_1 associates to PKCβI and PKCε and M_2 to PKCε

First, we studied how M_1 and M_2 mAChR affect PKCβI and PKCε isoforms at the NMJ. We used antibodies with high specificity for the corresponding protein at the predicted molecular weight: pPKCβI (Thr^{641}) 77 kDa, PKCβI 79 kDa, pPKCε (Ser^{729}) 90 kDa, PKCε 82 kDa (Figure 1A). The anti-pPKCβI antibody detected a second band corresponding to the PKC catalytic domain. This band was not affected by

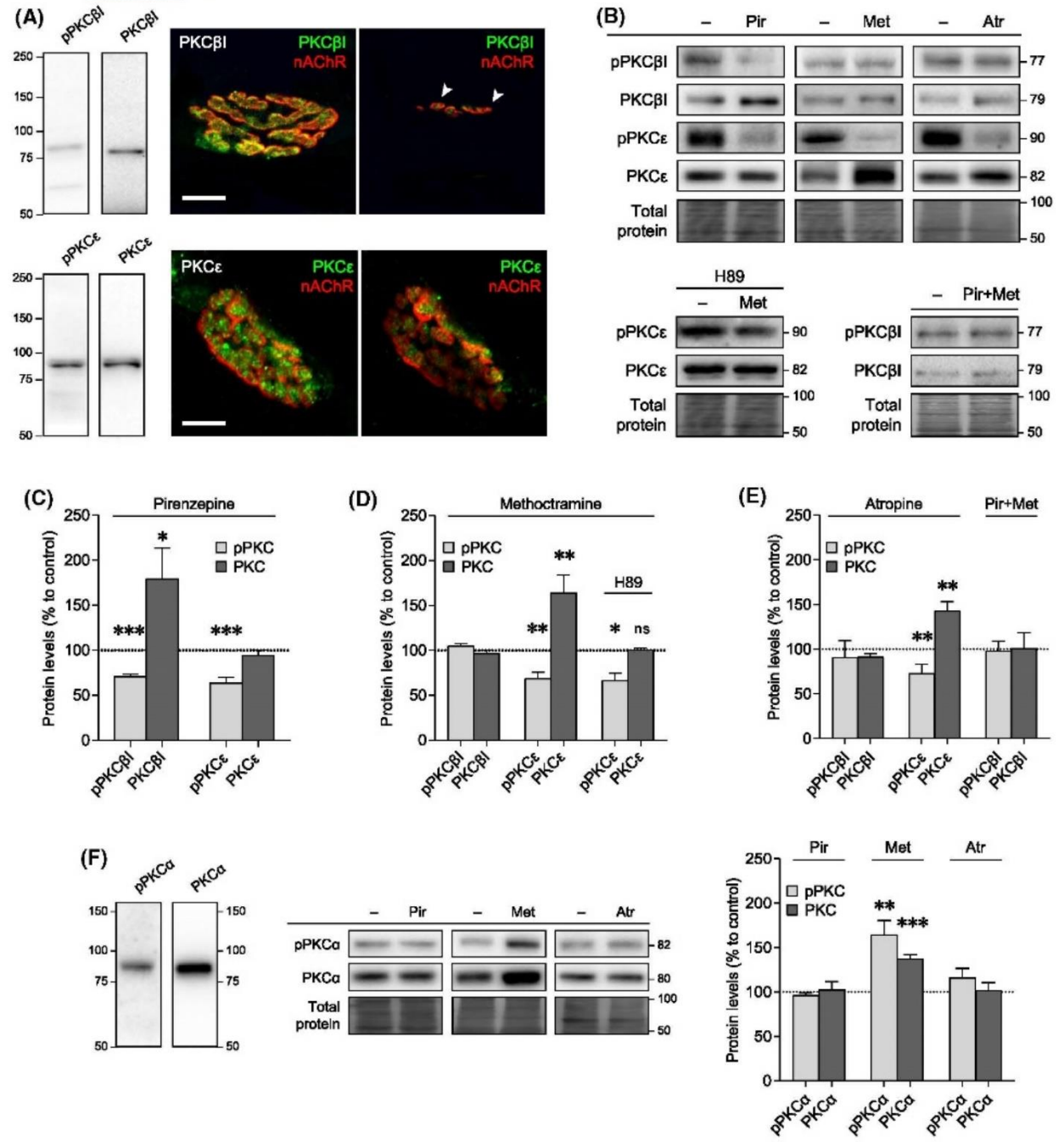

FIGURE 1 PKCβI, PKCε, and PKCα levels and maturation after muscarinic blockade. A, Molecular weight of the phosphorylated and total PKC βI isoform (pPKCβI and PKCβI) and PKC ε isoform (pPKCε and PKCε) at the rat diaphragm. Immunofluorescence-stained neuromuscular junctions of LAL muscle visualized at the confocal microscope. The images at the right are a confocal optical section of the left NMJ. NMJ with double labeling: α-bungarotoxin (α-BTX) conjugated with TRITC in red and PKCβI in green or PKCε in green. Scale bars = 10 μm. B, Western blot bands of PKC isoforms. C, Effect of M_1 inhibition (pirenzepine, Pir). D, Effect of M_2 inhibition (methoctramine, Met) and M_2 inhibition after a pretreatment with the PKA blocker H89. E, Effect of M_1/M_2 inhibition with atropine (Atr) and with a mixture of pirenzepine and methoctramine (Pir+Met). F, Molecular weight of the phosphorylated and total PKC α isoform (pPKCα and PKCα) at the rat diaphragm and effect of the muscarinic inhibitors. M_1 promotes the priming and activation of PKCβI whereas M_2 promotes PKCε. H89: PKA blocker. Data are expressed as percentage of protein levels after treatment. Mean value ± SD. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

the treatments and in 8% SDS-PAGE gels it could be well-distinguished from the predicted 79 kDa full-length pPKCβI band. The PKCβI and PKCε are exclusively presynaptic at the NMJ[22,23] and here we also show that their immunohistochemical staining is limited to the presynaptic region over the nicotinic acetylcholine receptors (nAChRs) (see arrows in the confocal section image of PKCβI) and does not surpass areas outside the NMJ endplate. The first two indicators of PKC activity that we studied were PKC phosphorylation, which indicates PKC priming, and protein level, which indicates PKC synthesis or activity-induced degradation (Figure 1B). PKC turnover is activity-dependent and can be used as marker of its activity.[17-20]

M_1 blockade with pirenzepine (Pir) decreased PKCβI Thr^{641} phosphorylation and increased its protein amount (Figure 1C). This increase in PKCβI levels along with the decrease in PKCβI priming could indicate an accumulation of the inactive kinase. Additionally, M_1 blockade decreased PKCε Ser^{729} phosphorylation without affecting its protein amount. Altogether, this suggests that M_1 signaling promotes the priming of both PKC βI and ε isoforms and probably reduces PKCβI levels due to activity-dependent degradation at basal conditions.

M_2 blockade with methoctramine (Met) did not affect PKCβI phosphorylation or its total levels (Figure 1D). Surprisingly, M_2 inhibition decreased the phosphorylation of PKCε and upregulated its total amount. Because the action of M_2 on pPKCε/PKCε is similar to that of M_1 on pPKCβI/PKCβI (Figure 1C-D), M_2 could be inducing the priming of PKCε and decreasing its protein level. To understand if M_2 acts directly on PKCε or it involves the classic mediator PKA, we used the inhibitor H89, a blocker of PKA catalytic subunits. A previous incubation with H89 before methoctramine abolished the increase in PKCε protein levels without altering the decrease in PKCε phosphorylation (Figure 1D). Altogether, these results show that M_2 signaling modulates PKCε rather than PKCβI, involving PKA to decrease PKCε levels and promoting PKCε phosphorylation through a mechanism that does not require PKA.

The combined action of M_1 and M_2 mAChR pathways can be determined with atropine (Atr), a well-known muscarinic pan-inhibitor. In particular, atropine has the same affinity for M_1 and M_2 and its effect on ACh release and PKA signaling is mimicked by a mixture of Pir +Met at NMJs.[2,53] The comparison between the effects of subtype-selective inhibitors versus atropine reveals which subtype has higher overall impact on each PKC isoform. Figure 1E shows that M_1/M_2 blockade with Atr did not affect PKCβI but increased the total levels of PKCε and decreased its phosphorylation. Compared to the results of the selective inhibitions, both M_1 and M_2 inhibitions are responsible for the downregulation of PKCε priming, whereas M_2 inhibition is responsible for the increase in PKCε protein levels. Interestingly, atropine does not replicate the effects of pirenzepine on PKCβI. This might suggest that M_1 inhibition needs an active M_2 to prime PKC βI and decrease its levels. In addition to Atr, we used a mixture of pirenzepine and methoctramine (Pir+Met) to check the implication of both M_1 and M_2 mAChRs over PKCβI. The Pir+Met treatment minimizes the participation of other mAChR subtypes (Figure 1E). Therefore, it is useful to check if the effect of atropine can be explained by mainly M_1 and M_2 blockade or, otherwise involves other mAChR subtypes. We found that Pir+Met incubation did not modify PKCβI protein levels and phosphorylation, mimicking atropine's effect. This reinforces that M_1 mAChR needs an active M_2 to modify PKCβI levels.

To check the scope of muscarinic signaling, we studied how muscarinic inhibition affects the PKCα isoform, which is mostly expressed at the post-synaptic muscle.[5,54,55] Unlike the presynaptic PKCβI and PKCε, pirenzepine and atropine did not affect PKCα (Figure 1F). However, M_2-blockade increased both PKCα protein levels and phosphorylation. This result indicates that M_2 signaling reduces PKCα priming and protein amount.

In summary, M_1 promotes the priming of PKCβI and PKCε and downregulates PKCβI probably through activation-induced degradation, which needs an active M_2. On the other hand, M_2 promotes PKCε priming and decreases its protein levels through PKA.

3.2 | M_1 translocates PKCβI and PKCε whereas M_2 only PKCε

Next, we studied how muscarinic signaling modulates the subcellular location of PKC isoforms in the membrane, which is a surrogate measure of PKC isoform activation.[56] To test the action of M_1 and M_2, we separated the membrane (particulate) and cytosolic (soluble) fractions of the samples after treatment. The membrane fraction contains detergent-insoluble compartments, including the plasma membrane, intracellular vesicles and other intracellular membranous compartments. We checked the fractionation purity with the cytosol marker GAPDH and the membrane marker Na^+/K^+-ATPase, which were highly enriched in their corresponding fraction and essentially undetectable on the opposite.

Figure 2 shows the distribution of the PKC isoforms between the membrane and cytosol. We set the value of control samples as 100% (membrane + cytosol) and calculated the treatment values in relation to the control. PKCβI and PKCε isoforms were more associated to the membrane regardless of their phosphorylation (proportion membrane–cytosol from control samples: pPKCβI 79-21% ± 4; PKCβI 79-21% ± 3; pPKCε 81-19% ± 5; PKCε 68-32% ± 5).

The effect of M_1 inhibition with Pir on PKCβI was limited to the membrane, decreasing its phosphorylation and

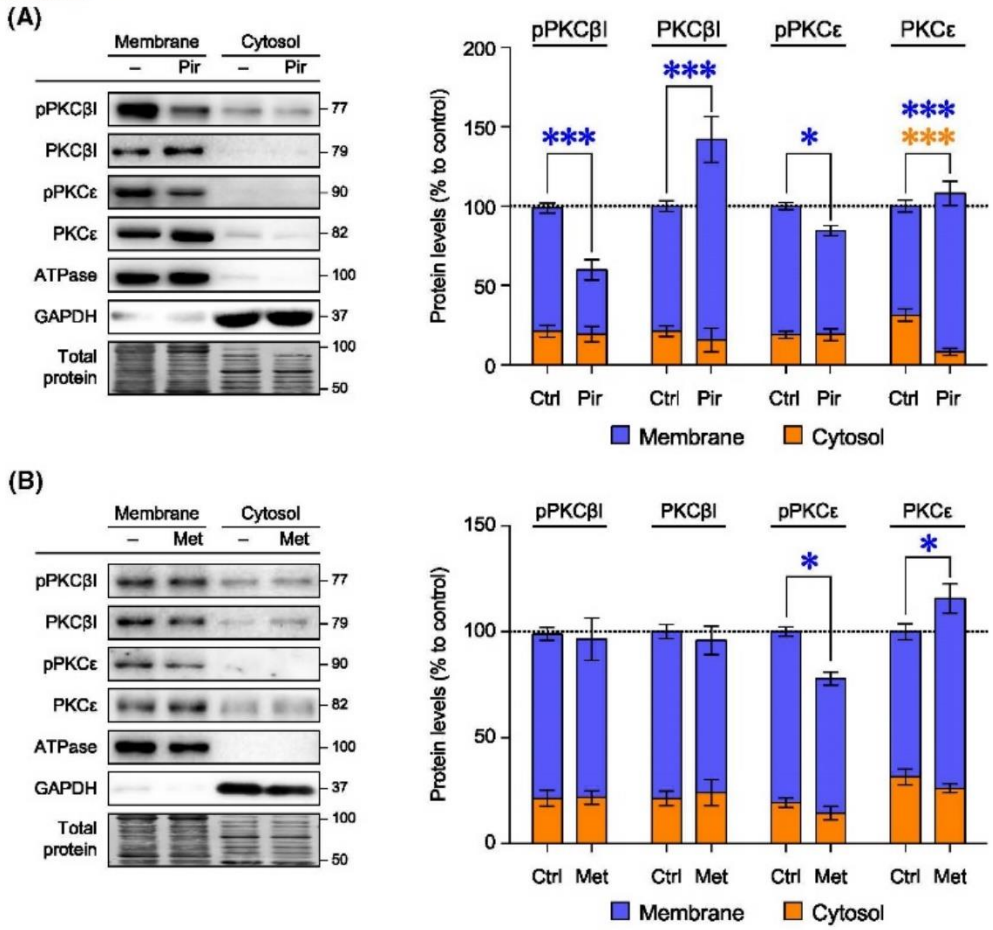

FIGURE 2 Distribution of the PKC isoforms between the membrane (particulated) and cytosol (soluble) fractions after muscarinic blockade. A, Western blot bands of PKC isoforms and effect of M_1 inhibition (pirenzepine, Pir). B, Western blot bands of PKC isoforms and effect of M_2 inhibition (methoctramine, Met). PKCβI membrane association is orchestrated by M_1 whereas that of PKCε is modulated by M_1 and M_2. Data are expressed as percentage of protein levels before and after treatment (mean value ± SD). Control value (Ctrl) is set at 100% (membrane + cytosol) and the treatment value is calculated in relation to the control. $*P < .05$, $***P < .001$

increasing the total protein levels (Figure 2A). The small amount of cytosolic PKCβI both before and after pirenzepine treatment makes translocation unlikely to explain the great increase in the membrane. Therefore, after M_1 blockade, PKCβI is accumulated on the membrane. Regarding PKCε, M_1 blockade decreased the phosphorylated PKCε in the membrane, accumulated total PKCε in the membrane and decreased it from the cytosol (see discussion) These results indicate that M_1 inhibition downregulates PKCε phosphorylation and induces its accumulation at the membrane.

M_2 inhibition with Met (Figure 2B) did not affect the subcellular distribution of pPKCβI or PKCβI, reinforcing the pervious conclusion that M_2 signaling does not involve PKCβI. Regarding PKCε, M_2 inhibition downregulated PKCε phosphorylation and increased its protein levels in the membrane fraction. In summary, M_2 blockade seems to inhibit the priming of PKCε and accumulate PKCε levels on the membrane.

3.3 | Both M_1 and M_2 activate PDK1

After observing that pirenzepine decreased the phosphorylation of PKCβI and PKCε and methoctramine decreased the phosphorylation of PKCε, we wondered if that was caused by an effect of muscarinic signaling over the priming of PKC. Thus, we studied the PKC-priming kinase PDK1 and its phosphorylation on Ser^{241} after M_1 and M_2 blockade. The antibodies detected a band of 60 kDa for both pPDK1 and PDK1 (Figure 3A). PDK1 is a synaptic kinase[15] and here we demonstrate that it is localized in the presynaptic region over postsynaptic nAChRs gutters (see the confocal section image at the right) and does not surpass areas outside the NMJ endplate.

The phosphorylation of PDK1 decreased after both pirenzepine and methoctramine treatments without affecting PDK1 protein levels (Figure 3B). The treatment with the pan-inhibitor atropine also decreased PDK1 phosphorylation without

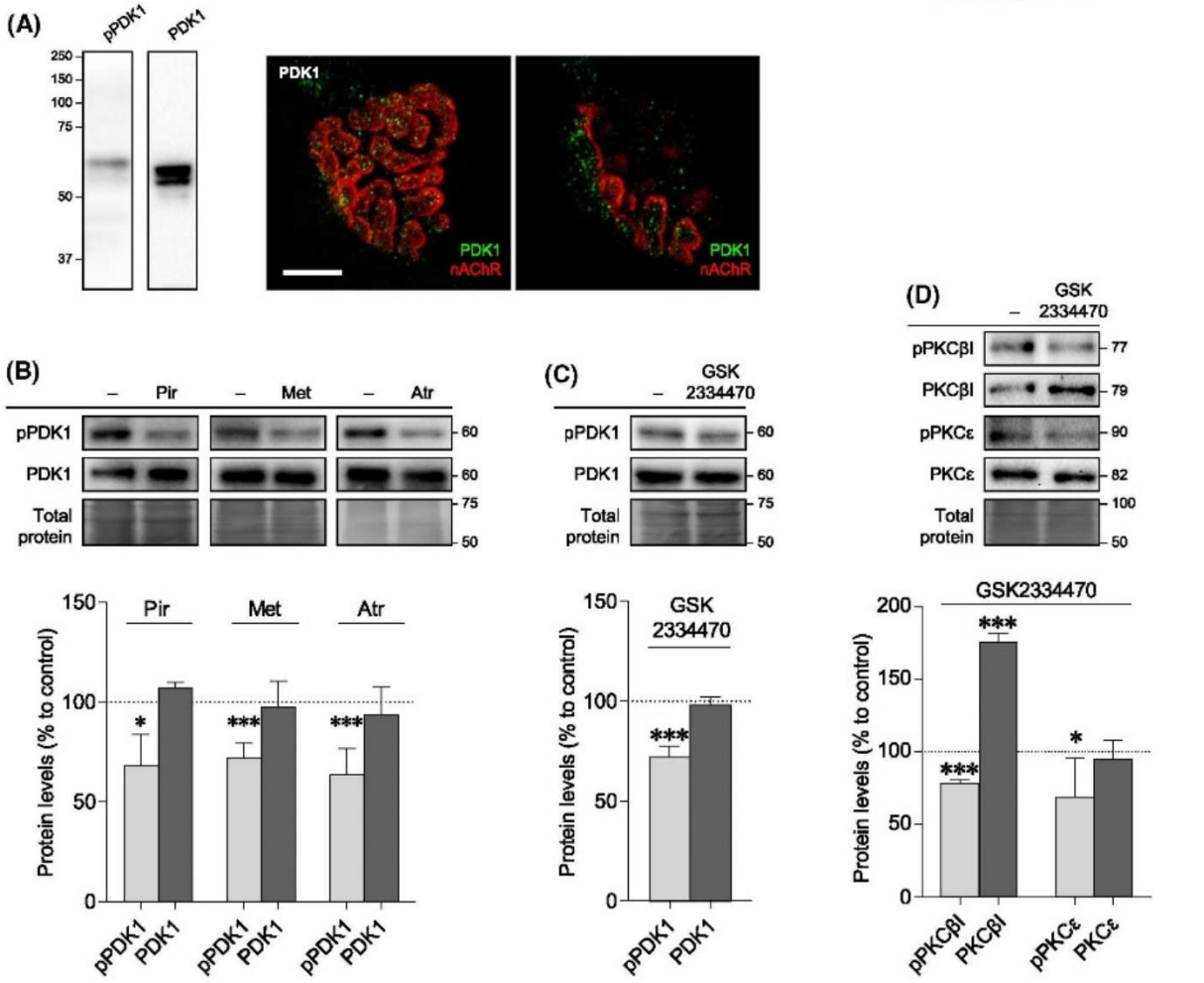

FIGURE 3 PDK1 protein levels and phosphorylation after muscarinic blockade. A, Molecular weight of the phosphorylated and total PDK1 (pPDK1 and PDK1) at the rat diaphragm. At the right: immunofluorescence-stained neuromuscular junctions of LAL muscle visualized at the confocal microscope. The images at the right are a confocal optical section of the left NMJ. NMJ with double labeling: α-bungarotoxin (α-BTX) conjugated with TRITC in red and PDK1 in green. Scale bars =10 μm. B, Effect of M_1 inhibition (pirenzepine, Pir), M_2 inhibition (methoctramine, Met) and M_1/M_2 inhibition (atropine, Atr). Both M_1 and M_2 pathways promote the phosphorylation of PDK1. C, Effect of PDK1 inhibition (GSK2334470) over the phosphorylated and total PDK1. D, Effect of PDK1 inhibition over the phosphorylated and total PKCβI and PKCε isoforms. Data are expressed as percentage of protein levels after treatment. Mean value ± SD. $^*P < .05$, $^{***}P < .001$

affecting PDK1 protein levels. This decrease in PDK1 phosphorylation correlates with the accompanying decrease in phospho-PKC previously detected (note that M_2 only affects PKCε; Figure 1D). To understand whether the signaling of both M_1 and M_2 mAChRs might be promoting PKC priming through PDK1, we studied the effect of PDK1 blockade. The specific PDK1 inhibitor GSK2334470 decreased the phosphorylation of PDK1 Ser^{241} without changing its protein levels (Figure 3C). Next, we tested whether PDK1 inhibition affects the downstream PKC isoforms. PDK1 inhibition decreased PKCβI phosphorylation and increased its protein levels (Figure 3D). PDK1 inhibition per se seems to reproduce the effect that M_1 inhibition with pirenzepine has over PKCβI. Regarding the PKCε isoform, PDK1 inhibition decreased its phosphorylation.

3.4 | M_1 and M_2 mAChR subtypes control PKC substrates

Because M_1 and M_2 mAChRs recruit specific PKC isoforms, they likely influence PKC substrates differently. Thus, we studied how M_1 and M_2 blockade affected the PKC targets Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}) and MARCKS ($Ser^{152/156}$) (Figure 4A-B).

M_1 inhibition downregulated the phosphorylation of all PKC substrates: Munc18-1 phospho-Ser^{313}, SNAP-25 phospho-Ser^{187} and MARCKS phospho-$Ser^{152/156}$ without affecting their protein amount (Figure 4C). Along with the decrease in the PKC-priming kinase PDK1, this result supports the interpretation that the changes observed over PKCβI and

PKCε after M_1 inhibition correspond to a decrease in PKC activity.

In opposition of M_1 inhibition, M_2 blockade increased the phosphorylation of some PKC substrates. This is the case of Munc18-1, whose phosphorylation and protein levels were increased by Met (Figure 4D). This suggests that M_2 signaling downregulates Munc18-1 synthesis, which possibly affects its phosphorylation levels. On the other hand, M_2 inhibits MARCKS phosphorylation, which is shown by M_2 inhibition upregulating MARCKS phosphorylation without changing its protein levels. In contrast, M_2 inhibition did not affect SNAP-25 Ser^{187} phosphorylation or its total protein levels, being the only substrate not affected by M_2 signaling.

Once determined the role of M_1 and M_2 subtypes per se, we proceeded to study their combined action on PKC substrates (Figure 4E). The M_1/M_2 inhibition with Atr decreased

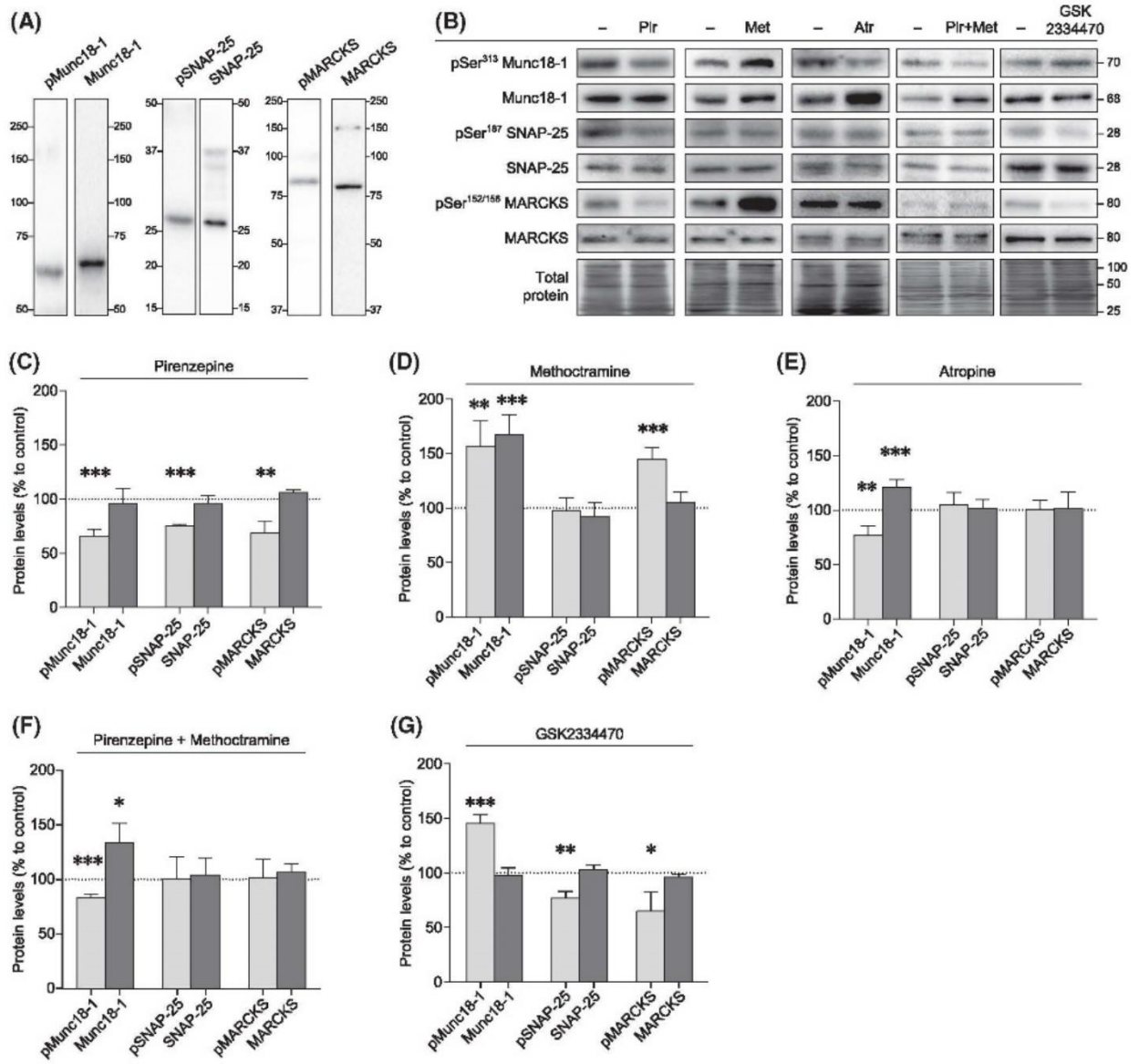

FIGURE 4 Munc18-1, SNAP-25, and MARCKS phosphorylation and protein levels after muscarinic blockade. A, Molecular weight of the phosphorylated and total Munc18-1 (pMunc18-1 and Munc18-1), SNAP-25 (pSNAP-25 and SNAP-25), and MARCKS (pMARCKS and MARCKS) at the rat diaphragm. B, Western blot bands of Munc18-1, SNAP-25 and MARCKS. C, Effect of M_1 inhibition (pirenzepine, Pir). D, Effect of M_2 inhibition (methoctramine, Met). E, Effect of M_1/M_2 inhibition (atropine, Atr). F, Effect of M_1/M_2 inhibition with a Pir and Met mixture (Pir+Met). G, Effect of PDK1 inhibition (GSK2334470). M_1 promotes the phosphorylation of all PKC substrates Munc18-1, SNAP-25, and MARCKS whereas the M_2 inhibits Munc18-1 levels and MARCKS phosphorylation. Both receptors converge on PDK1, whose activity promotes the phosphorylation of SNAP-25 and MARCKS and inhibits that of Munc18-1. Data are expressed as percentage of protein levels after treatment. Mean value ±SD. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

the phosphorylation of Munc18-1 and increased the total levels of Munc18-1. After comparing with the subtype selective inhibitors, M_1 inhibition seems responsible for the decrease in pMunc18-1, while the increase in Munc18-1 seems caused by M_2 inhibition. The M_1/M_2 inhibition with atropine did not change MARCKS phosphorylation or protein levels. This outcome on pMARCKS is in line with the action of Pir and Met and is possibly the result of the balance between the M_1-increasing and M_2-decreasing actions. Moreover, SNAP-25 $pSer^{187}$ remained unaltered after M_1/M_2 blockade. Interestingly, atropine does not replicate the effect of pirenzepine, which may indicate that M_1 needs M_2 active to promote SNAP-25 phosphorylation. Alternatively, atropine could affect other receptors like M_4 mAChR which could counter the action of M_1. To rule out the effect of atropine on other muscarinic receptors, we tested the effect of a mixture of pirenzepine and methoctramine (Pir + Met) (Figure 4F). An incubation with Pir + Met replicated the same results as atropine over Munc18-1, SNAP-25 and MARCKS, reinforcing the participation of solely the M_1 and M_2 mAChR subtypes.

Finally, because we determined that M_1 and M_2 selective blockers downregulate PDK1 activity at the NMJ, we analyzed whether the inhibition of PDK1 per se could also affect the downstream PKC substrates (Figure 4G). PDK1 inhibition with GSK2334470 increased the phosphorylation of Munc18-1 without affecting its protein amount. On the other hand, PDK1 inhibition decreased the phosphorylation of SNAP-25 $pSer^{187}$ and MARCKS $pSer^{152/156}$ without affecting their total levels. These data show that PDK1 blockade replicates the effect of M_2 inhibition over Munc18-1 and the effect of M_1 inhibition over SNAP-25 and MARCKS, reinforcing that PDK1 is at the crossroad between M_1 and M_2 signaling over PKC.

3.5 | M_1 signaling involves PKCβI and PKCε to phosphorylate Munc18-1, MARCKS, and SNAP-25

To determine the M_1 and M_2 mAChR pathways more accurately, we investigated the involvement of PKC isoforms by studying if the specific competitive peptides βIV_{5-3} (inhibitor of PKCβI) and εV_{1-2} (inhibitor of PKCε) could prevent the effects of the selective muscarinic blockades. Also, because we detected that PKA was involved in the regulation of M_2 on PKC and some PKC substrates have PKA phosphorylation sites (see discussion), we also used H89 to determine the PKA role.

A previous incubation with βIV_{5-3} or εV_{1-2} before pirenzepine (Figure 5A) abolished the effect of M_1 blockade on Munc18-1 phosphorylation (Figure 5B). This indicates that both PKCβI and PKCε are necessary for M_1 mAChR action on Munc18-1 at the NMJ. In contrast, H89 did not prevent pirenzepine from reducing Munc18-1 phosphorylation, which indicates that PKA does not participate in M_1/Munc18-1 phosphorylation.

All previous inhibition of PKCβI, PKCε or PKA prevented pirenzepine from reducing SNAP-25 phosphorylation (Figure 5C). This indicates that M_1 mAChR action on SNAP-25 needs both PKC and PKA kinases, suggesting that it is more controlled than Munc18-1 phosphorylation.

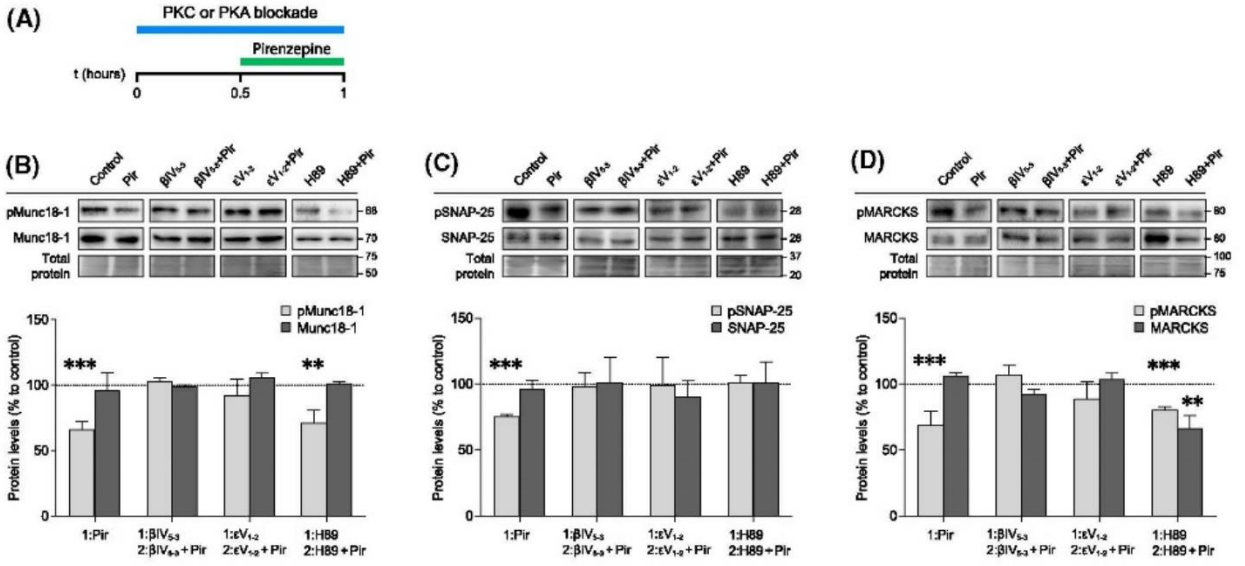

FIGURE 5 Effect of PKC and PKA inhibitors on the action of M_1 over PKC substrates. A, The specific inhibitors of PKCβI (βV_{5-3}), PKCε (εV_{1-2}), and PKA (H89) were pre-incubated before M_1 blockade (pirenzepine, Pir) to determine the requirement of each kinase. B, Western blot bands and optical densitometry of phosphorylated (pMunc18-1) and total Munc18-1. C, Western blot bands and optical densitometry of phosphorylated (pSNAP-25) and total SNAP-25. D, Western blot bands and optical densitometry of phosphorylated (pMARCKS) and total MARCKS. Data are mean values ±SD. $^{**}P < .01$, $^{***}P < .001$

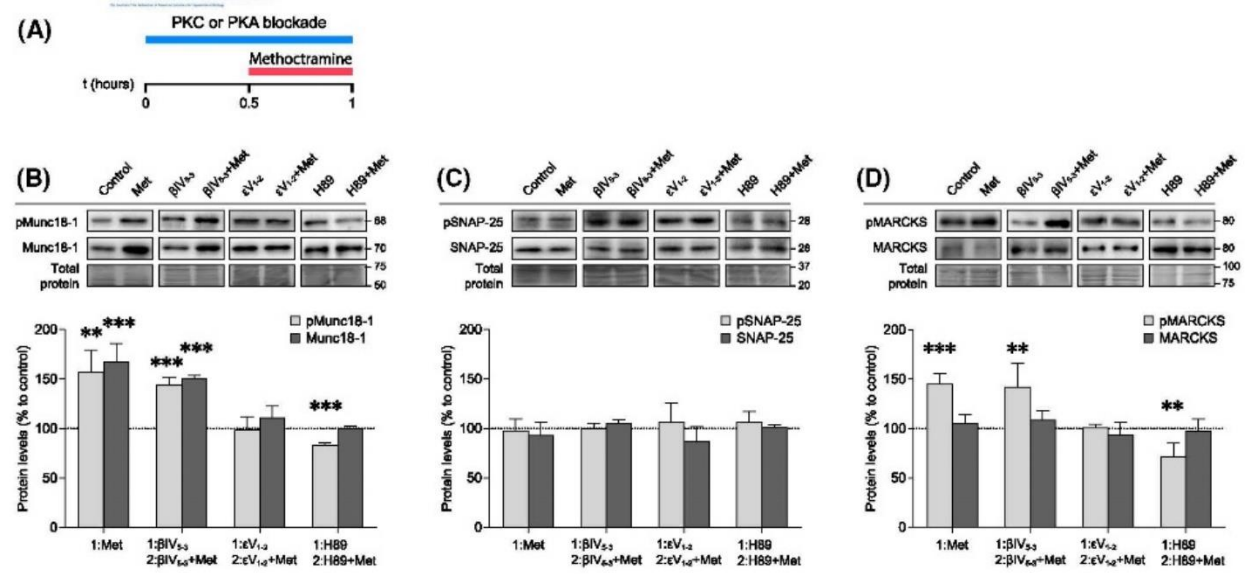

FIGURE 6 Effect of PKC and PKA inhibitors on the action of M_2 over PKC substrates. A, The specific inhibitors of PKCβI (βV_{5-3}), PKCε (εV_{1-2}), and PKA (H89) were pre-incubated before M_2 blockade (methoctramine, Met) to determine the requirement of each kinase. B, Western blot bands and optical densitometry of phosphorylated (pMunc18-1) and total Munc18-1. C, Western blot bands and optical densitometry of phosphorylated (pSNAP-25) and total SNAP-25. D, Western blot bands and optical densitometry of phosphorylated (pMARCKS) and total MARCKS. Data are mean values ±SD. $^{**}P < .01$, $^{***}P < .001$

Finally, the previous incubation with βIV_{5-3} abolished the downregulation of pirenzepine on MARCKS phosphorylation without modifying MARCKS protein levels (Figure 5D). A previous blockade of PKCε also prevented pirenzepine from affecting MARCKS phosphorylation and protein levels. This indicates that M_1 mAChR action on MARCKS needs both PKCβI and PKCε. In contrast, deleting PKA activity with H89 did not prevent pirenzepine from reducing MARCKS phosphorylation. Surprisingly, we found a decrease in MARCKS levels. Because PKA is involved in protein translation (see discussion), shutting down PKA from the system might be revealing a MARCKS-degrading pathway induced by M_1 mAChR.

3.6 | M_2 signaling involves PKCε and PKA to reduce Munc18-1 level and MARCKS phosphorylation

We previously found that M_2 blockade upregulated Munc18-1 levels and phosphorylation, probably by enhancing Munc18 synthesis and, hence, its phosphorylation levels. The PKCβI isoform is dispensible for this mechanism, because a previous incubation of the PKCβI inhibitor βIV_{5-3} did not prevent the effect of methoctramine (Figure 6A-B). Instead, Munc18-1 upregulation was abolished by a previous inhibition of PKCε (εV_{1-2}) and PKA (H89). This indicates that M_2 mAChR requires PKCε and PKA, but not PKCβI, to decrease Munc18-1 at the NMJ. In line with the previous results, where M_2 inhibition did not affect SNAP-25, none of the PKC and PKA inhibitors added any effect on the methoctramine treatment (Figure 6C).

We also studied the PKC and PKA role on the upregulation of phospho-MARCKS after M_2 inhibition (Figure 6D). Previously blocking PKCβI did not prevent M_2 modulation, because Met still increased MARCKS phosphorylation. However, a previous inhibition of PKCε (εV_{1-2}) and PKA (H89) abolished the upregulation of phospho-MARCKS after M_2 inhibition. This indicates that M_2 mAChR requires PKCε and PKA, but not PKCβI, to decrease pMARCKS at the NMJ. Interestingly, without PKA, M_2 blockade decreased Munc18-1 and MARCKS phosphorylation, which is coincident with the decrease in phospho-PKCε on the same conditions (Figure 1D).

3.7 | Muscarinic subtypes induce the translocation of Munc18-1, SNAP-25, and MARCKS

We investigated the distribution between the membrane and cytosol of Munc18-1, MARCKS and SNAP-25 after muscarinic inhibition to clarify the implications of the previous regulations (Figure 7). At basal conditions, Munc18-1 was strongly associated to the membrane, both in its phosphorylated form pMunc18-1 (membrane–cytosol, 85-15% ± 4) as well as its total levels (76-24% ± 3). Similarly, pSNAP-25 Ser^{187} was also associated to the membrane (77-23% ± 3) as well as total SNAP-25

(77-23% ± 4). Phosphorylated MARCKS was equally distributed between the two fractions (55-45% ± 5) and MARCKS slightly more to the cytosol (38-62%±6).

The decrease in Munc18 phosphorylation after M_1 inhibition was located at the membrane fraction (Figure 7A). This reduction in pMunc18-1 was accompanied by a translocation of the total Munc18-1 species from the membrane to the cytosol. The decrease in SNAP-25 phosphorylation after M_1 inhibition occurred at the membrane fraction. However, this decrease did not affect the distribution of the total SNAP-25. Similar to SNAP-25, M_1 inhibition also decreased MARCKS phosphorylation on the membrane compartment without affecting the distribution of the total MARCKS. Altogether, these results indicate that M_1 signaling promotes Munc18 (Ser^{187}) phosphorylation and its association to the membrane. M_1 also induces the phosphorylation of SNAP-25 and MARCKS on the membrane without affecting their membrane translocation.

M_2 inhibition with Met increased the phosphorylation of Munc18-1 in the membrane without affecting it on the cytosol (Figure 7B). Additionally, M_2 inhibition also upregulated the total protein Munc18-1 levels in the membrane fraction rather than in the cytosol. In consistency with the previous results, the blockade of M_2 did not affect SNAP-25 Ser^{187} phosphorylation and it did not induce the translocation of its protein levels. Finally, the increase in MARCKS phosphorylation by Met was concentrated in the membrane fraction without affecting the phosphorylation on the cytosol. This M_2 modulation did not affect the total levels of MARCKS, which remained unchanged in both the membrane fraction and in the cytosol.

3.8 | The synaptic region contains the M_2 signaling on PKCβI, PKCε, Munc18-1, and SNAP-25

To study the muscarinic signaling that occurs at the presynaptic terminal, we selected PKC isoforms that participate in neurotransmitter release and are exclusively expressed at the presynaptic terminal of the NMJ.[5,22,24] However, it is not possible to isolate the phrenic nerve and preserve its

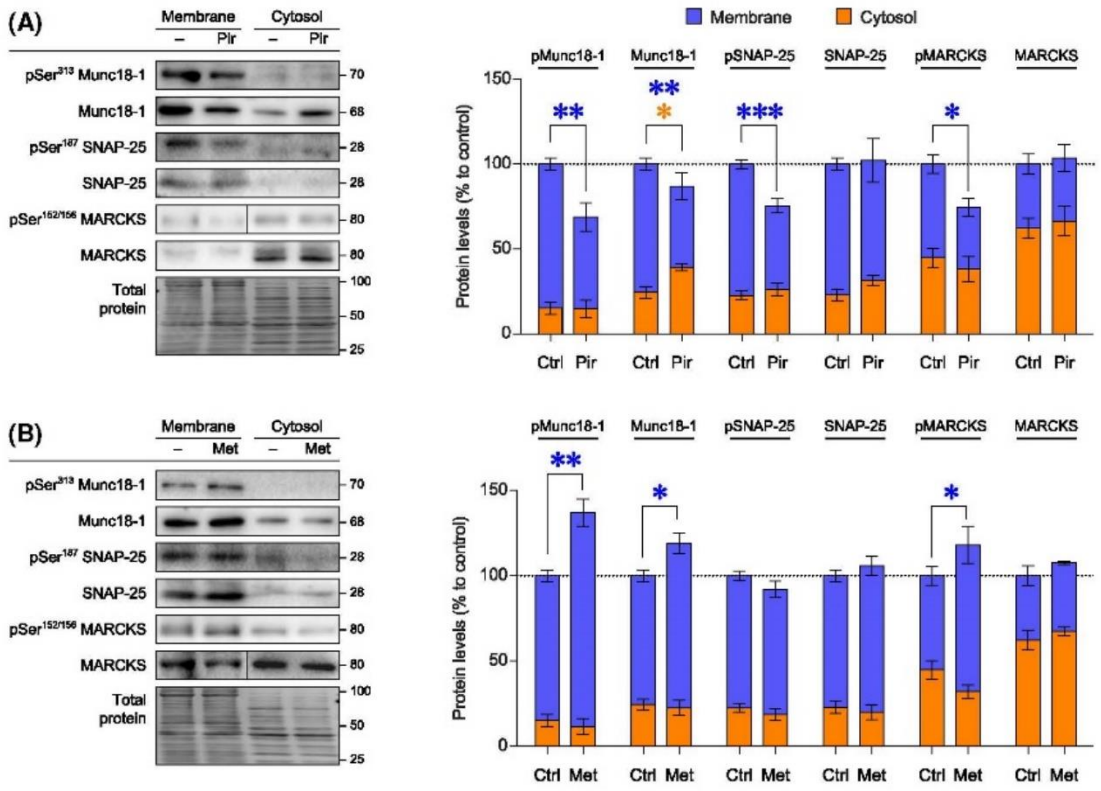

FIGURE 7 Distribution of the PKC substrates between the membrane (particulated) and cytosol (soluble) fractions after muscarinic blockade. A, Western blot bands and optical densitometry of PKC substrates after M_1 blockade (pirenzepine, Pir). B, Western blot bands and optical densitometry of PKC substrates after M_2 blockade (methoctramine, Met). M_1 promotes Munc18 phosphorylation and translocation to the membrane. Additionally, M_1 induces SNAP-25 and MARCKS phosphorylation on the membrane without affecting their translocation. Data are expressed as percentage of protein levels before and after treatment (mean value ± SD). Control value (Ctrl) is set at 100% (membrane + cytosol) and the treatment value calculated in relation to the control. $^*P < .05$, $^{**}P < .01$. $^{***}P < .001$

physiological conditions, so the biochemical studies must be carried out in whole muscle samples. Indeed, we show that M_2 signaling can affect post-synaptic proteins like PKCα. Thus, to better define the location of the presynaptic PKCβI and PKCε signaling in this study, we separated the synaptic-enriched region of the hemidiaphragms from the extrasynaptic regions. This can be done by adding α-bungarotoxin conjugated to TRITC to the medium at the last 10 minutes of treatment and then dissecting under the microscope the tissue region positive in AChRs (synaptic region) from the region without AChR-marking (extrasynaptic region) (Figure 8A). Although α-BTX-TRITC is added in a non-blocking low concentration, the results could be influenced by the presence of this nicotinic AChR blocker (see Discussion). As expected, PKCβI, PKCε, Munc18-1, SNAP-25, and their phosphorylated forms were enriched in the synaptic region and significantly lower in the extrasynaptic region (Figure 8B-F). The presence of these molecules in the extrasynaptic region could be due to their presence in axon branches, muscle spindles and, feasibly, distant or non-stained NMJs that cannot be excluded from the extrasynaptic region. M_2 blockade did not affect PKCβI phosphorylation or its total levels (Figure 8C). On the other hand, the effect of methoctramine over PKCε was restricted at the synaptic region, reducing PKCε phosphorylation and upregulating PKCε total protein levels (Figure 8D). The levels of PKCε at the extrasynaptic region were lower than at the synaptic region and methoctramine did not induce any effect. With regard to Munc18-1, methoctramine increased its protein levels and phosphorylation in the synaptic region (Figure 8E). Although Munc18-1 was enriched in the synaptic region of the diaphragm, this molecule was more abundant than the others in the extrasynaptic area (around 65% versus control values). In contrast, SNAP-25 was more specific for the synaptic region than Munc18-1 and, in concordance with the whole muscle samples, it was not affected

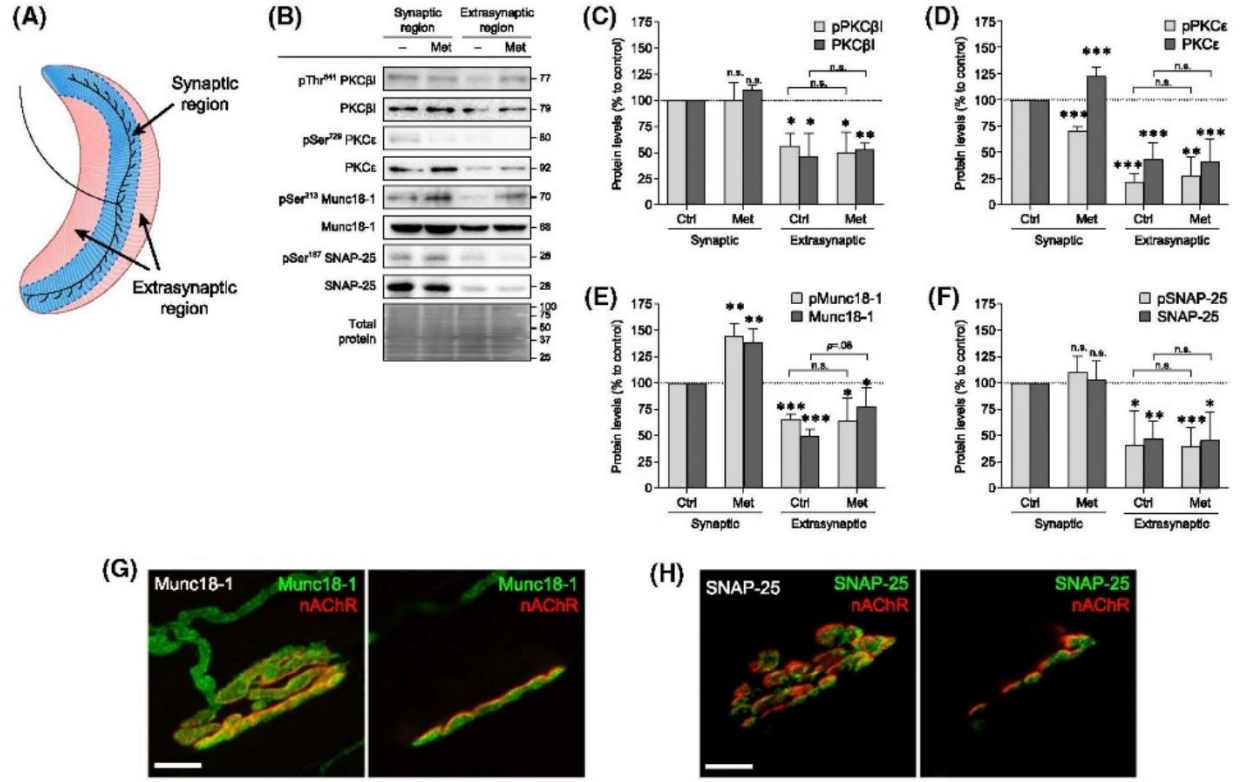

FIGURE 8 Synaptic localization of the effects of M_2 signaling. A, Representation of the synaptic and extrasynaptic regions of the rat diaphragm. B, Western blot bands of the phosphorylated and total levels of PKCβI, PKCε, Munc18-1, and SNAP-25 in the synaptic and extrasynaptic regions of the diaphragm before and after M_2 blockade (methoctramine, Met). C–F, Effect of M_2 blockade over the phosphorylation and total levels of (C) PKCβI, D, PKCε, (E) Munc18-1, and (F) SNAP-25. G–H, immunofluorescence-stained neuromuscular junctions of LAL muscle visualized at the confocal microscope. The images at the right are a confocal optical section of the left NMJ. NMJ with double labeling: α-bungarotoxin (α-BTX) conjugated with TRITC in red and Munc18-1 in green (G) and SNAP-25 in green (H). Scale bars = 10 μm. Data are expressed as percentage of protein levels before and after treatment (mean value ± SD). The control value (Ctrl) from the synaptic region is set at 100% and the rest are calculated in relation to the control. n.s. not significant, $*P < .05$, $**P < .01$, $***P < .001$

by methoctramine (Figure 8F). In concordance to the presence of Munc18-1 at the extrasynaptic region, Figure 8G shows that Munc18-1 is expressed at the presynaptic component of the NMJ and also in nerve terminals, which extend outside the synaptic region. The optical section shows that Munc18-1 immunostaining is concentrated at the presynaptic component, over the nAChR postsynaptic gutters (Figure 8G right). SNAP-25 was exclusively located in the presynaptic component of the NMJ (Figure 8H). The optical section shows that SNAP-25 green immunolabeling is concentrated over the nAChR postsynaptic gutters (Figure 8H right). Altogether, these results demonstrate the effect of M_2 blockade on PKCβI, PKCε, Munc18-1, and SNAP-25 is associated to the synaptic area of the diaphragm, reinforcing that this particular signaling occurs at the presynaptic terminal.

4 | DISCUSSION

The M_1 and M_2 muscarinic receptor subtypes induce opposed outcomes on ACh release at the NMJ. M_1 increases whereas M_2 decreases the end-plate potential.[1] Interestingly, both subtypes couple PKC to neurotransmission when inhibited with selective blockers such as pirenzepine or methoctramine. The M_1 and M_2 muscarinic receptors are specifically expressed at the nerve terminal and Schwann cells,[57] where they likely modify several PKC isoforms. Until now, it was unknown if muscarinic receptors had different preference for particular PKC isoforms. In this study, we selected the PKCβI and PKCε as representatives of classical and novel PKC families because they are exclusively expressed at the presynaptic terminal [21-23] and participate in synaptic transmission.[22,24,31,34,38] A summary of the main findings is represented in Figure 9.

4.1 | M_1 signaling on PKC

The M_1 muscarinic signaling potentiates ACh release at many cholinergic synapses, including the neuromuscular junction.[1,53,58] M_1 muscarinic receptors are linked to G_q protein signaling and PLCβ activation.[59] In turn, PLCβ activates PKC through the production of inositol 1,4,5-triphosphate (IP_3), which mobilizes Ca^{2+}, and the phorbol ester diacylglycerol.[60] Then, neurotransmitter release is enhanced through PKC, which phosphorylates numerous targets of the exocytotic machinery, but also by other phorbol ester-sensitive proteins like Munc13.[26,61,62]

Our results show that M_1 signaling promotes the maturation (priming) of both PKCβI and PKCε isoforms. This could

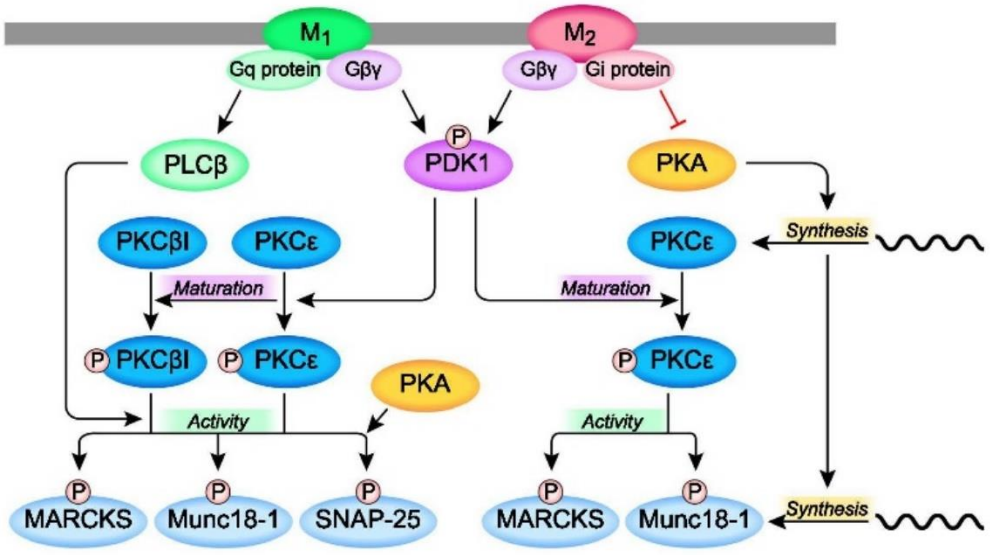

FIGURE 9 Summary of M_1 and M_2 muscarinic regulation of PKC signaling at the NMJ. The M_1 mAChR promotes the phosphorylation of PDK1 and the maturation of the presynaptic PKC isoforms PKCβI and PKCε at the membrane compartment of the NMJ. M_1 also activates PKC isoforms through PLCβ, causing PKCβI protein degradation at the membrane and PKCε release to the cytosol (see Discussion for the role of PDK1 on PKC translocation). This signaling pathway triggers the PKC phosphorylation of Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}), and MARCKS ($Ser^{152/156}$) and their recruitment to the membrane (SNAP-25 phosphorylation by M_1 requires PKA activity). In parallel to M_1, the M_2 mAChR signaling also promotes the phosphorylation of PDK1 and the priming of the PKC isoform PKCε at the membrane. However, M_2 downregulates PKCε protein levels at the membrane through a PKA-dependent pathway. This inhibition extends to the PKC substrates Munc18-1 and MARCKS. If the PKA-dependent pathway of M_2 is blocked, M_2 signaling is still able to promote PKCε maturation, which enhances Munc18-1 and MARCKS phosphorylation, similarly to M_1 signaling. Therefore, M_1 and M_2 muscarinic receptors balance PKCβI and PKCε priming, protein levels, and activity on the mediators of the synaptic vesicle release machinery

be mediated by PI3K/PDK1 because M_1 signaling activates PI3K in rat hippocampal neuron cultures[63] and we accordingly found that M_1 increases PDK1 Ser^{241} phosphorylation and PDK1 promotes both PKCβI and PKCε phosphorylations. On the other hand, M_1 mAChR regulates differently the levels of PKCβI and PKCε isoforms. It downregulates PKCβI in the total and in the membrane fraction but does not affect PKCε total protein levels and translocates it to the cytosol. Several reasons indicate that M_1 might be inducing PKCβI degradation after activation rather than inhibiting PKCβI synthesis. First, M_1 associates to the G_q protein and PKC activation, which leads to PKC activity-dependent degradation.[17-20] In concordance, we previously found at the NMJ that PKCβI degradation occurs after being activated by phrenic nerve stimulation to potentiate ACh release.[5,22] Another finding supporting that M_1 mAChR triggers PKCβI activity and its turnover is that the PKC downregulation occurs at the membrane fraction, the compartment where studies in cell cultures found activity-dependent PKCβI ubiquitination and degradation.[17] Additionally, we found that M_1 mAChR requires PKCβI activity to induce the phosphorylation of the SNARE regulator Munc18-1 (Ser^{313}), the SNARE core protein SNAP-25 (Ser^{187}) and the cytoskeleton-related MARCKS ($Ser^{152/156}$). In relation to Munc18-1 and PKCβI, experiments with the same βI inhibitor used here demonstrated that PKCβI is also necessary for the increase of Munc18-1 phosphorylation caused by nerve stimulation but not for the increase of Munc18-1 levels.[31] Also, the enhance of Munc18-1 PKC-phosphorylation is closely related to neurotransmission[26] and adds to our knowledge about how M_1 enhances acetylcholine release at the NMJ.[1] On the other hand, SNARE complex formation and neurotransmission is also regulated by SNAP-25 PKC-phosphorylation.[33,36,64] Our results show that M_1 mAChR uses PKCβI to phosphorylate SNAP-25, reinforcing the previously demonstrated role of the βI isoform on the neurotransmission at the NMJ as the inhibition of PKCβI decreases the size of end-plate potentials.[22] M_1 promotion of PKC phosphorylation over MARCKS has been determined in literature.[40] Multiple PKC isoforms can phosphorylate MARCKS in vitro and in vivo.[23,65] Here, we determine that, at the NMJ, M_1 requires the presynaptic PKCβI to phosphorylate MARCKS. Additionally, we found that the phosphorylation of Munc18-1, SNAP-25 and MARCKS by M_1 signaling occurs at the membrane and that M_1 also induces the translocation of Munc18-1 from the cytosol to the membrane. These changes in the membrane are concordant with an activation of all three substrates, which is closely related to their membrane association.[33,66,67]

Contrary to PKCβI, M_1 inhibition did not affect PKCε protein levels. This distinct behavior between PKCβI and PKCε could be because PKCε is less sensitive to activity-dependent degradation. Indeed, PKCε rate of downregulation is 3-fold slower than other PKC isotypes.[68] Alternatively, PKCε activity-dependent degradation can be modulated by other PKC isoforms.[69] In this regard, we know that the inhibition of PKCβI in basal conditions downregulates PKCε levels at the neuromuscular junction.[31] Thus, the blockade of M_1/PKCβI with pirenzepine could reduce PKCε and counter an accumulation of PKCε levels after M_1 inhibition. The PKCε isoform is crucial to facilitate and trigger multiple mechanisms involved in ACh release at the NMJ.[23,24,31,38] This isoform generally participates in the first signaling steps of multiple receptors, acting like an early kinase of various signaling cascades.[24] This is also the case for muscarinic signaling, where PKCε inhibition prevents mAChRs from modulating ACh release if it is performed before, but PKCε inhibition does not influence the NMJ release after mAChR blockade.[24] In concordance, here we inhibited PKCε before muscarinic blockade to demonstrate the dependency of M_1 on PKCε activity at the molecular level. Here we found that M_1 muscarinic signaling uses PKCε to phosphorylate the three substrates Munc18-1, SNAP-25, and MARCKS, demonstrating the facilitatory role of this kinase for M_1. In concordance, PKCε activity has been previously linked to the phosphorylation of Munc18-1,[31] SNAP-25,[38] and MARCKS.[23,65] The results also show that M_1 inhibition decreases the primed pPKCε form and enhances total PKCε in the membrane. We interpret this result as that, after M_1 inhibition, PKCε isoform is not allowed to perform its facilitating role and therefore it does not undergo activity-induced degradation at the membrane,[17-20,23,38] resulting in an accumulation of the inactive isoform in the membrane. Altogether, these molecular results support the previous electrophysiological findings showing that PKCε is necessary for M_1 to increase ACh release.[24] Because the membrane fraction that we studied contains the insoluble membrane compartments like the plasma membrane, synaptic vesicles, and mitochondria, it is possible that inhibited PKCε becomes more associated to the cytoskeleton (insoluble fraction)[70,71] or translocates between different cell compartments.[72] On the other hand, PKC priming by PDK1 acts on cytoskeleton-associated PKC enzymes (in the insoluble membrane fraction) and releases them to the cytosol.[70,71] Therefore, it is possible that the PDK1 inhibition by pirenzepine contributes to the association of total non-phosphorylated PKCε to the membrane fraction (in this case associated to the insoluble cytoskeleton). In addition, it should not be discarded that the increase of PKCε in the membrane after Pir could induce some activity on another substrate which we did not study here. Previous work at the NMJ suggested that the treatment with pirenzepine, although it downregulates IP_3/Ca^{2+} signaling and PKC activity, also couples at least some PKC isoform to participate in ACh release.[1] Our research drives us to think that this isoform could be PKCε acting on different p-substrates than Munc18-1, MARCKS, and

SNAP-25, as it seems to integrate different receptor signaling pathways at the NMJ[23,24,31,38] and here we find that it is recruited to the membrane. Together with our translocation data, the translocation of PKCε to the membrane after M_1 inhibition could be responsible for the coupling of PKC after M_1 inhibition. However, further research on the role of PKCε and its targets is needed to comprehend this complex mechanism. On the other hand, it is not surprising that both PKCβI and PKCε could phosphorylate these substrates, considering that all PKC isoforms show a high degree of sequence similarity in their kinase domain.[65] It is very interesting that both PKCβI and ε isoforms are necessary to phosphorylate these substrates, but they do not replace themselves (necessary but not sufficient). In coincidence, the activity of the novel PKCε isoform is a prerequisite necessary for classic PKC activity at the NMJ.[24] This remarks the importance of studying the differences and cooperation between PKC isoforms.

PKA activity is essential for maintaining neurotransmission at the NMJ and it has recently been linked with M_1 muscarinic signaling.[2,63] Here we show that M_1 signaling does not require PKA activity to promote the PKC phosphorylation of Munc18-1. However, PKA participates in Munc18-1 expression and we discuss it in the next section about M_2 signaling. In regard to MARCKS, PKA does not promote the M_1/PKC phosphorylation but it maintains its protein levels. This is because, without PKA activity, M_1 blockade caused a decrease in MARCKS levels. PKA is involved in protein translation and shutting down PKA from the system might be revealing a MARCKS-degrading pathway induced by M_1 mAChR inhibition. Interestingly, M_1/PKC phosphorylation of SNAP-25 Ser^{187} requires PKA activity. PKA is known to phosphorylate SNAP-25 on Thr^{138}.[37,73] We previously determined that blocking the neuromuscular PKA with H-89 decreases SNAP-25 Thr^{138} phosphorylation.[2] Together with the present results, this might indicate that PKA SNAP-25 Thr^{138} favors PKC phosphorylation on SNAP-25 Ser^{187}.

Therefore, at the neuromuscular junction, M_1 activates PDK1 and induces the maturation of the classical PKCβI and the novel PKCε. Besides, M_1 uses G_q/PLCβ and calcium mobilization to trigger PKCβI activity and its consequent degradation. M_1 needs PKCε activity to facilitate the M_1 downstream signaling. Both PKC isoforms regulate the phosphorylation of Munc18-1, MARCKS, and SNAP-25 substrates (the latter with the help of PKA), which associate to the membrane and participate in neurotransmission at the NMJ and other cellular processes.

4.2 | M_2 signaling on PKC

The M_2 muscarinic signaling reduces the neurotransmission from cholinergic synapses, including the neuromuscular junction.[1,53] M_2 receptors are generally linked to Gi proteins, which inhibit adenylate cyclase and block PKA activity by downregulating cAMP production.[58] One of the consequences we recently demonstrated is that M_2 signaling decreases the PKA phosphorylation of SNAP-25 Thr^{138} at the NMJ.[2] Besides the PKA pathway, further studies revealed that PKC activity is also necessary for the M_2 muscarinic signaling in various neuromuscular models.[1,74,75]

In the present work, we studied how M_2 influences the PKC isoforms that are exclusive of the presynaptic terminal.[21-23] In particular, we show that M_2 signaling does not affect the classical PKCβI isoform priming, levels or subcellular distribution and this PKC isoform is not required for any substrate phosphorylation affected by M_2 blockade that we studied. In concordance, a previous study in portal vein myocytes shows that M_2 recruits novel instead of classical PKC isoforms.[76] Our study concurs with this idea, because we found that the novel PKCε isoform participates in many M_2 downstream regulations at the NMJ.

M_2 signaling induces PKCε phosphorylation priming at the membrane fraction as well as the phosphorylation of the PKC master kinase PDK1, in the same way as M_1. This could be linked to M_2/PI3K activation through the Gβγ subunit[74,76] and here we demonstrate that PDK1 promotes PKCε phosphorylation. On the other hand, M_2 signaling downregulates PKCε protein levels in the membrane fraction. These modulations of PKCε and pPKCε by M_2 occur at the synaptic region of the hemidiafragm, in consistency with the presynaptic location of the isoform.[21,23] As mentioned before, the increase of PKC levels can be interpreted as an accumulation of the inactive kinase or, on the contrary, as more synthesis. M_2 activity is unlikely to induce PKCε activity and degradation, as the PKCε isoform is not very sensitive to activity-dependent degradation[68] and M_2 inhibition activates and couples PKC to participate in acetylcholine release at the NMJ.[1] Besides, evidence demonstrate that PKCε is active. We observed that M_2 blockade induces PKC activity, upregulating Munc18-1 PKC-phosphorylation, Munc18-1 protein levels and MARCKS PKC-phosphorylation. In concordance, PKCε activity at the NMJ upregulates both Munc18-1 phosphorylation and protein levels[31] and phosphorylates MARCKS.[23] Also, here we verified that PKCε activity is required for M_2 blockade to upregulate Munc18-1 and MARCKS. Thus, the increase in PKCε by M_2 inhibition is consistent with enhanced PKC activity and it is possibly caused through protein synthesis (see below for the implication of PKA).

To identify the components of M_2/PKCε pathway, we investigated the effect of PKA inhibition. Our results show that M_2 blockade uses PKA activity to increase PKCε levels but does not need PKA to decrease PKCε phosphorylation. This reveals that M_2 signaling affects PKCε through two different pathways: a PKA-dependent pathway where M_2 downregulates PKCε, and a PKA-independent pathway where

M_2 induces PKCε maturation through phospho-PDK1. The mentioned PKA-independent pathway is concordant with the previous studies linking M_2 and novel PKC through the Gβγ subunit and PI3K activation,[74,76] hence does not need G_i protein and PKA activity. On the other hand, M_2 reduces PKCε levels via G_i protein and PKA inhibition and, when M_2 is blocked, PKA activity increases and upregulates PKCε. The phosphorylation of PKCε does not increase along with the total level because the PDK1-induced phosphorylation remains downregulated by Met. Because the PKCε isoform is exclusively expressed at the synaptic terminal of NMJs,[24] where gene expression does not take place, the upregulation of its protein levels might be caused by a posttranscriptional mechanism. Concordantly, PKA activity has been associated to the modulation of mRNA translation through the phosphorylation of the eukaryotic elongation factor 2 kinase, inducing a general reduction of mRNA translation but increasing the translation rate of a small subset of synaptic proteins.[77,78] Also, PKA promotes the translation of various proteins via phosphorylation of polypyrimidine tract-binding protein 1.[79]

Regarding the span of M_2 signaling, this mAChR subtype is specifically expressed at the nerve terminal and Schwann cells.[57] Here we describe a molecular pathway confined in the nerve endings, because the participating PKCβI and PKCε are exclusively presynaptic[5,22,23] and the end-targets are Munc18-1 and SNAP-25, neurotransmision modulators unique to the nerve endings.[31,38] We observed that M_2 signaling only affects the synaptic region of the hemidiaphragm, where both PKC isoforms, Munc18-1 and SNAP-25 were enriched. Munc18-1 was quite detectable in the extrasynaptic area (around 65% versus control value). This is in line with our previous report that Munc18-1 is also present in the nerve axons,[31] which stretch outside the synaptic region. In fact, when studying the effect of methoctramine in the extrasynaptic region, we observed a tendency to increase Munc18-1 protein levels ($P = .8$) that was not significant. However, muscarinic signaling may extend further than the presynaptic terminal and affect postsynaptic proteins. Here we show that M_2 mAChR downregulates the levels and priming of PKCα, an isoform which is mainly expressed at the postsynaptic site. M_2 mAChR at the NMJ induces an auto-inhibitory feedback over ACh release and our data suggest that M_2 signaling on PKCα could be linked to a reduction of the postsynaptic responsiveness to ACh. Other findings showing that synaptic events influence pre- and post-synaptic kinases are that mAChR signaling controls the widely expressed PKA[2] and that phrenic-induced activity under blocked postsynaptic contraction induces the degradation of the presynaptic PKCβI and the post-synaptic PKCβII.[5]

In this study we found that M_2 does not affect SNAP-25 Ser^{187} phosphorylation by PKC at the NMJ. Indeed, M_2 signaling rather regulates SNAP-25 through the PKA-phosphorylation at Thr^{138}.[2] Here, we also demonstrate that PKA activity is required for M_2 to modulate Munc18-1 and MARCKS. Interestingly, we found that all PKC phosphorylations inhibited by M_2, and hence attributed to PKCε, require PKA activity. This further supports the idea that M_2/PKA pathway modulates upstream the activity of PKCε over those substrates,[23,31] which provide a molecular clue to the functional results about the PKA-dependent pathway on M_2 outcomes.[80] Interestingly, we also found that a previous inhibition of PKA shifts M_2 to perform an M_1-like signaling in relation to Munc18-1 and MARCKS phosphorylation. This is because after H89 preincubation, M_2 blockade decreases both Munc18-1 and MARCKS phosphorylation which is the result of M_1 inhibition. This is concordant with previous functional studies that showed when PKA is previously inactivated, blocking either M_1 or M_2 leads to a similar reduction in transmitter release.[1] Indeed, this result unmasks the PKA-independent pathway described before where M_2 induces PKCε phosphorylation. In this, M_2 blockade would downregulate PKCε phosphorylation through PDK1 (our results) and Gβγ/PI3K[74,76] and, without PKA activity in the system, this PKC inhibition would decrease Munc18-1 and MARCKS phosphorylation.

4.3 | Muscarinic balance on PKC at the NMJ

We examined the joint action of M_1 and M_2 receptors with the pan-muscarinic inhibitor atropine. We found that atropine affects PKCε but not PKCβI, a similar effect to inhibit M_2 with the selective inhibitor methoctramine. Atropine's affinity is practically the same for both M_1 and M_2 mammalian subtypes,[58] which could indicate that M_2 inhibition has a greater overall effect on the protein levels of PKC than M_1. Concordantly, previous studies showed that atropine increases ACh release at the NMJ in the same way as methoctramine.[1,53] Alternatively, the fact that atropine does not replicate the effect of M_1 inhibition on PKCβI protein levels might indicate that M_1 needs an active M_2 to modulate PKCβI turnover.

The upregulation of PKCε by atropine can be explained by the opposite action of M_1 and M_2. On the one hand, atropine blocks M_1 thus decreasing PKCε activity and the phosphorylation of its substrate Munc18-1 (discussed in more detail below). However, the accompanying M_2 blockade by atropine also liberates PKA activity, which upregulates PKCε levels and buffers the action of M_1. This M_1/M_2 counter regulation probably balances PKCε activity and protects the synapse from an excessive or insufficient PKC activity.

Regarding PKC priming, atropine decreases the phosphorylation of the upstream kinase PDK1, indicating that the overall action of M_1 and M_2 muscarinic receptors activates PDK1 at the NMJ. Interestingly, atropine causes the

same decrease in PDK1 levels as the decrease observed when M_1 or M_2 are inhibited separately. The lack of additive effect suggests that both mAChR subtypes could be using the same pathway. As mentioned before, both M_1 and M_2 muscarinic receptors have been shown to activate PI3K,[63,74,76] which could be a common mechanism to activate PDK1. We previously demonstrated that PKC priming phosphorylation at the NMJ is enhanced by presynaptic stimulation and that the resulting muscle contraction increases it further,[22] suggesting a postsynaptic retrograde regulation. However, tropomyosin-related kinase B (TrkB) receptor signaling was not responsible for this priming and, contrarily, it rather acted downregulating PKCα and PKCβI phosphorylation levels.[22] That suggested the existence of a different pathway, activated by neuromuscular activity, which was promoting PKC phosphorylation and compensating TrkB downregulation. In the current study, we found that M_1 and M_2 muscarinic receptors promote PDK1 activity as well as PKC maturation at the NMJ. These receptors, whose action is triggered by the ACh released at the NMJ, could explain how neuromuscular activity enhances PKC maturation in an activity-dependent manner.

Atropine action over the PKC activity can be observed by studying PKC substrates. In this study we observed that atropine downregulates Munc18-1 phosphorylation, increases Munc18-1 protein levels, and it does not affect the levels or phosphorylation of SNAP-25 and MARCKS. Comparing these results with the selective inhibitions suggests that M_1 blockade is responsible for the decrease in phospho Munc18-1, via PKCβI and PKCε, whereas M_2 blockade is responsible for the increase in Munc18-1 protein levels, via PKCε and PKA. Therefore, the activities of both mAChRs balance each other: M_1 promotes Munc18-1 phosphorylation whereas M_2 signaling controls the levels of this regulatory synaptic protein. Regarding SNAP-25, it is a key synaptic molecule which is difficult to modulate with treatments due to the multiple mechanisms finely regulating it.[2,37,38,81] Interestingly, here we show that M_1 blockade decreases SNAP-25 Ser^{187} phosphorylation, although we could not observe this effect with the general muscarinic inhibitor atropine. This suggests that M_1 signaling needs M_2 active to promote SNAP-25 PKC-phosphorylation. Interestingly, the PKA phosphorylation of SNAP-25 (Thr^{138}) follows a similar regulation, where M_2 downregulates it and needs the activity of M_1.[2] If the inhibitory M_2 signaling on PKA/SNAP-25 is necessary for the M_1/PKC phosphorylation of SNAP-25, the current results might indicate that PKA hinders the action of PKC over SNAP-25. However, further research is needed to shed light on this complex mechanism and clarify the complementary role of PKC and PKA on SNAP-25. Regarding the third substrate examined, the absence of effect over MARCKS phosphorylation is probably the result of the counter regulation between M_1 and M_2: the decreasing effect of M_1 blockade and the increasing effect of M_2 blockade probably cancel each other out. MARCKS phosphorylation is related to PKCε[23] and, concordantly, here we demonstrated that both mAChR subtypes modulate PKCε and require its activity to modulate MARCKS.

As a pan-muscarinic inhibitor, atropine also inhibits other mAChR subtypes like M_3, M_4 and M_5. However, studies at the adult NMJ show that mainly M_1 and M_2 orchestrate the NMJ neurotransmitter release,[53,82-84] whereas other mAChR subtypes like M_3 and M_4 only participate during the development of the newborn NMJ.[83,85,86] M_1 and M_2 implication can be determinated with a mixture of Pir+Met. In the frog NMJ, this mixture fully mimmicks the effects of atropine on neurotransmitter release[53] and in rat hippocampal place cells, where the mixture replicated the effects of scopolamine, another known pan-muscarinic inhibitor.[87] As expected, we observed that Pir + Met incubation causes the same effects as atropine over PKCβI, Munc18-1, SNAP-25, and MARCKS protein levels and phosphorylation, reinforcing the major participation of M_1 and M_2 in the muscarinic signaling.

Finally, the role of PDK1 activity at the NMJ also demonstrates the balance between M_1 and M_2 receptors. Overall, PDK1 blockade per se has effects similar to M_1 inhibition, regulating in an equal manner PKCβI, PKCε, and the substrates SNAP-25 and MARCKS. This supports that M_1 signaling relies mainly on the PKC pathway, where PDK1 activity plays an important role. On the other hand, M_2 blockade also inhibits PDK1 activity and we observed that PDK1 blockade per se induces effects similar to M_2 inhibition over Munc18-1 phosphorylation. Interestingly, atropine does not induce the same modulations as PDK1 inhibition. This is probably because PDK1 inhibition only disrupts PKC priming, one step of PKC activation, whereas atropine induces a pan-muscarinic inhibition, involving both the PKC pathway—including PDK1—and the PKA pathway.

4.4 | Conclusion and future prospects

The present results demonstrate a signaling pathway that M_1 and M_2 mAChRs use to regulate neurotransmission. The M_1 mAChR signaling promotes the phosphorylation of PDK1 and the priming of the presynaptic PKC isoforms PKCβI and PKCε at the NMJ, which occurs at the membrane compartment. On the same subcellular compartment, M_1 activation of PKC induces PKCβI protein degradation and displaces PKCε from the membrane, without changing PKCε total protein levels. This signaling pathway triggers the PKC phosphorylation of Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}), and MARCKS ($Ser^{152/156}$) and their recruitment to the membrane. On the other hand, the M_2 mAChR signaling also promotes the phosphorylation of PDK1 and the priming of PKCε at the membrane compartment. However, M_2

signaling downregulates PKCε protein levels at the membrane compartment through a PKA-dependent pathway. This inhibition extends to the PKC substrates Munc18-1 and MARCKS. Interestingly, when PKA is blocked, M_2 signaling is able to promote Munc18-1 and MARCKS phosphorylation like M_1 signaling. The complementary activities of M_1 and M_2 muscarinic receptors balance PKCβI and PKCε priming, protein levels, and activity on mediators of the synaptic vesicle release machinery. Altogether this provides for the first time a molecular clue of M_1 and M_2 muscarinic and PDK1/PKC regulation of neurotransmitter release at the NMJ.

In this work we have identified a signaling pathway specific of the presynaptic motoneuron. This has been possible because we selected PKC isoforms and targets that participate in neurotransmitter release and are exclusively expressed at the presynaptic terminal of the NMJ.[21-23] We performed several tests to verify the location of the signaling in this study: (i) we confirmed by immunohistochemistry the presynaptic location of these molecules at the NMJ, (ii) we demonstrated that the effect of M_2 blockade on PKCβI, PKCε, Munc18-1, and SNAP-25 is associated to the synaptic area of the diaphragm, reinforcing that this particular signaling occurs at the presynaptic terminal, and (iii) we checked the effect of mAChR antagonists over the PKCα isoform, which is preferentially expressed in the postsynaptic muscles, showing the communication between cells through the mAChR signaling and the complexity of the mAChR regulation in the tripartite cellular NMJ.

Altogether, our observations provide in vivo examples of muscarinic modulation in a physiological model. Identifiying how muscarinic inhibitors and PKC isoforms participate in neurotransmission is important for prospective therapies. For instance, drugs in development depend upon the balance between the various isoenzymes present.[88] PKC isoforms are key for neurotransmitter release at the NMJ and are affected in symptomatic and presymptomatic stages of amyotrophic lateral sclerosis.[89,90] The dual modulation of M_1 and M_2 mAChRs could be used to readjust neuromuscular function and preserve neuromuscular function and muscle strength being useful for muscular paralysis, fall prevention, aging, and neuromuscular disorders such as amyotrophic lateral sclerosis and Duchenne muscular dystrophy.

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CONFLICT OF INTEREST

The authors declare no competing interests.

ETHICS STATEMENT

The animals were cared for in accordance with the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All the procedures realized were revised and authorized by the Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 0289).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MAL, JT, NG. Performed the experiments: VC, LJ, AP, MD, MT. Analyzed the data: VC, MAL, JT. Wrote the paper: VC, NG, MAL, JT.

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CHAPTER 5

GENERAL DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
MUSCARINIC RECEPTOR MODULATION OF PROTEIN KINASE A, PROTEIN KINASE C AND EXOCYTOTIC PROTEINS AT THE NEUROMUSCULAR JUNCTION
Víctor Cilleros Mañé

CHAPTER 5

GENERAL DISCUSSION

Structural Outline

*In **Chapter Five – General Discussion**, the publications are combined and interpreted together. Sections 1 and 2 describe, respectively, the M_1 and the M_2 mAChR signaling. Both of these sections elaborate on the crosstalk between pathways. Section 3 links our results to the existing literature on neurotransmitter secretion at the NMJ. Finally, Section 4 argues the value of such findings within the framework of neuromuscular health.*

Neuromuscular junctions are the structures where the motor nervous system communicates with muscles. The communication is bidirectional, with presynaptic neurons controlling muscle contraction and, in turn, myocytes sending retrograde signals to promote the neuron's survival and tune neurotransmission (Gomez-Pinilla *et al.*, 2008). Neurotransmission is the cornerstone of this interaction and it is fundamental for the health of the musculoskeletal system. However, its molecular modulation remains poorly understood, and so do potential approaches that could prevent NMJ degeneration. This thesis has focused on the basic science of muscarinic autoreceptors. These receptors are excellent candidates of the nerve-muscle interplay because they are presynaptic, sense the released ACh and adjust the amplitude of the end-plate potential (Slutsky *et al.*, 1999; Santafé *et al.*, 2003; Garcia *et al.*, 2005).

The M_1 and M_2 muscarinic receptors compete to bind ACh. **Their activity is constitutive**: present at basal conditions (Seifert and Wenzel-Seifert, 2002) and enhanced by spontaneous ACh release (Losavio and Muchnik, 1997, 2000). The actions of M_1 and M_2 antagonistically auto-regulate neurotransmission at the NMJ. M_1 increases the ACh release whereas M_2 decreases it (Santafé *et al.*, 2003). In this thesis, we studied their signaling and how they modulate the downstream kinases PKA and PKC to regulate the synaptic release machinery at the NMJ (Santafé *et al.*, 2003).

1. M_1 muscarinic signaling at the NMJ

The **M_1 muscarinic signaling enhances the neurotransmission** in many cholinergic synapses, including the neuromuscular junction (Caulfield and Birdsall, 1998; Slutsky *et al.*, 1999; Santafé *et al.*, 2007). At the NMJ, an imbalance of muscarinic signaling **couples PKC to participate and potentiate neurotransmission** (Santafé *et al.*, 2003, 2007). To our knowledge, the M_1-derived coupling of PKC to ACh release at the NMJ has only been observed by electrophysiology. Therefore, we aimed to obtain precise molecular details of that mechanism. Here we review our results about M_1 signaling at the NMJ, how it modulates specific isoforms of PKC, its targets of the synaptic release machinery (Cilleros-Mañé *et al.*, 2021), and how M_1 cross-modulates the M_2/PKA pathway (Cilleros-Mañé *et al.*, 2020).

1.1. The muscarinic M_1/PKC pathway at the NMJ

The M_1/PKC signaling that we have studied at the NMJ is **located in the presynaptic terminal** (■ Figure 16-A). First, this location is set by the expression of muscarinic receptors, which is highly associated with synapses in innervated myocytes (Reyes and Jaimovich, 1996; Liu *et al.*, 2002; Furlan and Godinho, 2005; Garcia *et al.*, 2005; Wright *et al.*, 2009). Furthermore, we concretized our study by selecting presynaptic proteins. In particular, these were the PKC isoforms PKCβI and PKCε, the PDK1 kinase and the targets Munc18-1 and SNAP-25, all of them proteins that are exclusively presynaptic at the NMJ (Perkins *et al.*, 2001; Obis *et al.*, 2015a; Hurtado *et al.*, 2017a, 2017b) and involved in neurotransmission (Morgan *et al.*, 2005; Obis *et al.*, 2015b; Hurtado *et al.*, 2017a; Simó *et al.*, 2018, 2019).

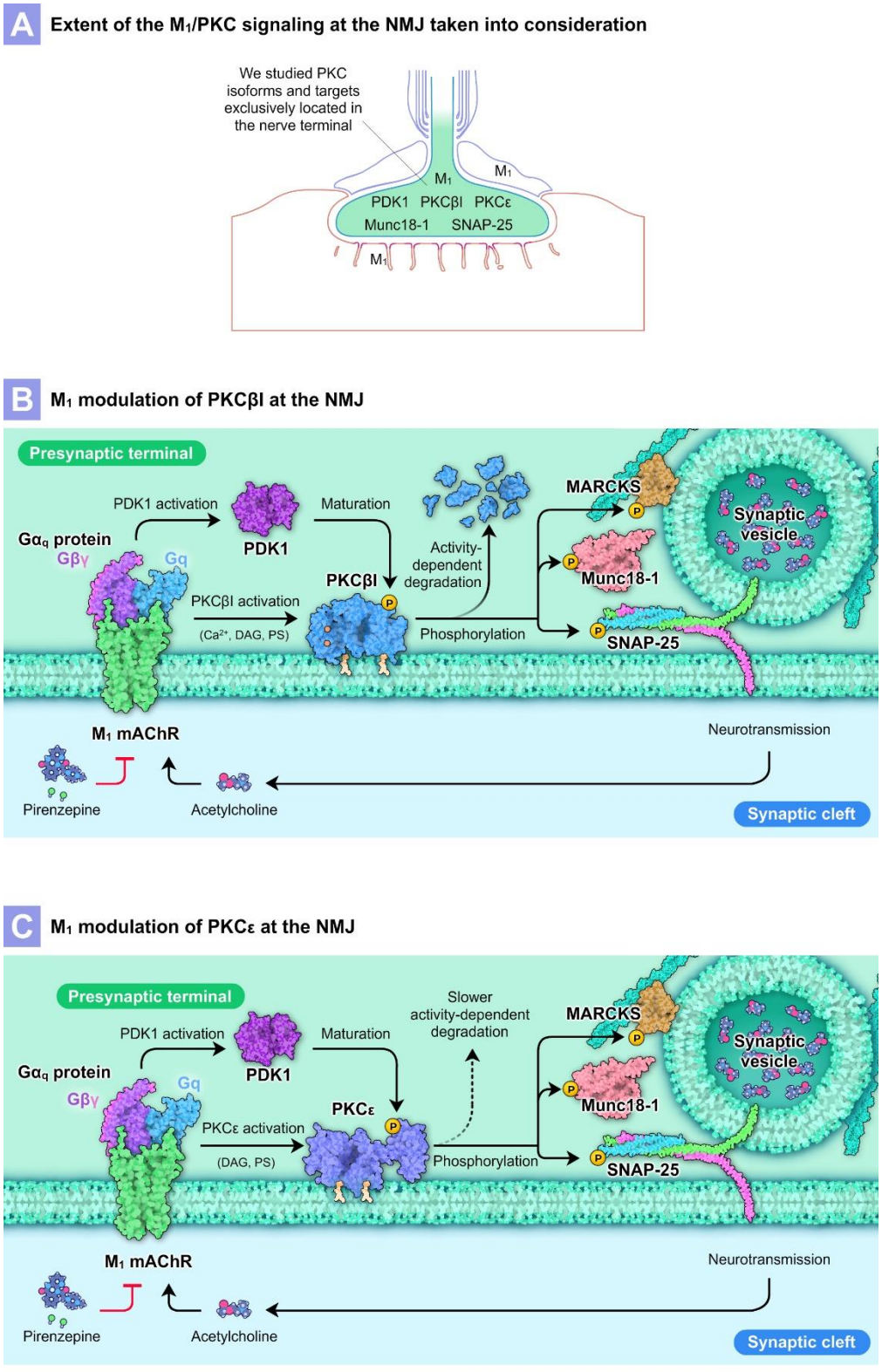

Figure 16. **M_1 muscarinic signaling over the PKC pathway at the NMJ.** A We concretized our study of the presynaptic branch of M_1/PKC signaling by selecting molecules that were exclusive of nerve terminals. B M_1 activates PDK1 and uses Gq/PLCβ and calcium mobilization to trigger PKCβI activity and its consequent degradation. C M_1 similarly activates PKCε but without degradation. Both PKC isoforms regulate the phosphorylation of Munc18-1, MARCKS and SNAP-25 which associate with the membrane and participate in neurotransmission at the NMJ and other cellular processes. *Source: own elaboration.*

PKC maturation by PDK1 is one of the first steps a PKC must undergo to become competent to respond to second messengers is its maturation (Shirai and Saito, 2002; Newton, 2010; Hurtado *et al.*, 2017b). We found that **M_1 mAChR promotes the autophosphorylation of PDK1 on Ser^{241}** (■ Figure 16-B). This phosphorylated Ser^{241} is essential for and enhances PDK1 activity (Casamayor *et al.*, 1999). Then, we demonstrated that PDK1 activity was required for the M_1-mediated maturation of both PKCβI and PKCε (Cilleros-Mañé *et al.*, 2021). To our knowledge, this was the first report of direct PDK1 modulation by muscarinic signaling. The link between M_1 and PDK1 activation could be PI3K, because it generates the phosphatidylinositol molecules required for PDK1 activation and M_1 signaling has been recently shown to activate PI3K in rat hippocampal neuron cultures (Zhao *et al.*, 2019). We previously reported that presynaptic stimulation and muscle contraction enhance PKC maturation (Hurtado *et al.*, 2017a). The TrkB receptor was not responsible, as it rather downregulated PKCα and PKCβI phosphorylation (Hurtado *et al.*, 2017a). That suggested the existence of a different pathway, activated by neuromuscular activity, that could promote PKC phosphorylation and compensate TrkB downregulation. In the current thesis, we found that **muscarinic receptors promote PDK1 activity as well as PKC maturation at the NMJ**. Overall, it is endearing to think that muscarinic receptors might be responsible for the PKC priming during presynaptic stimulus and muscle contraction at the skeletal muscle. We are currently testing this idea in our laboratory (Polishchuk *et al.*, in preparation).

Another mechanism to modulate PKC is changing its protein levels. We found that M_1 mAChR downregulates the PKCβI protein levels in whole-cell diaphragm lysates and at the membrane fraction. Several reasons suggested that **M_1 induces PKCβI activation and activity-dependent degradation** (■ Figure 16-B) rather than an inhibition of its synthesis. First, M_1 classically activates PKC, which leads to activity-dependent degradation (Parker *et al.*, 1995; Lee *et al.*, 1996; Lu *et al.*, 1998; Kang *et al.*, 2000). In concordance, PKCβI degradation at the NMJ occurs after other activating stimuli (Besalduch *et al.*, 2010; Hurtado *et al.*, 2017a). Secondly, the PKCβI turnover mediated by M_1 occurs at the membrane fraction, the compartment where *in*

vitro studies found activity-dependent PKCβI ubiquitination and degradation (Lu *et al.*, 1998). Additionally, our data shows that **PKCβI activity is required for M_1 signaling to enhance the PKC phosphorylation** of the SNARE regulator Munc18-1 (Ser^{313}), the SNARE core protein SNAP-25 (Ser^{187}) and the cytoskeleton-related MARCKS ($Ser^{152/156}$) (■ Figure 16-B). The PKC phosphorylations of Munc18-1 and SNAP-25 are associated with increased neurotransmission (Kataoka *et al.*, 2000; Barclay *et al.*, 2003; Shu *et al.*, 2008; Katayama *et al.*, 2017) and are further discussed in the Section 3 of this discussion.

On the other hand, **M_1 inhibition did not change PKCε protein amount** (■ Figure 16-C). This distinct behavior between PKCβI and PKCε is probably because PKCε is less sensitive to activity-dependent degradation. Indeed, PKCε rate of downregulation is 3-fold slower than other PKC isotypes (Olivier and Parker, 1992). On the other hand, PKCε activity-dependent degradation is also buffered by other PKC isoforms (Goode *et al.*, 1995). At the NMJ, PKCβI inhibition in basal conditions downregulates PKCε (Simó *et al.*, 2018). Therefore, M_1/PKCβI inhibition with pirenzepine could counter an accumulation of inactive PKCε. PKCε is essential to facilitate multiple mechanisms that promote ACh release at the NMJ (Obis *et al.*, 2015a, 2015b; Simó *et al.*, 2018, 2019). Indeed, PKCε acts upstream of several pathways (Obis *et al.*, 2015b). For example, blocking PKCε prevents muscarinic drugs from modulating ACh release (Obis *et al.*, 2015b). Here we also inhibited PKCε before muscarinic blockade to investigate the dependency of M_1 and PKCε at the molecular level. We observed that **M_1 muscarinic signaling requires an active PKCε to phosphorylate the three substrates** Munc18-1, SNAP-25 and MARCKS, demonstrating the facilitatory role of this kinase for M_1.

Surprisingly, we observed that **M_1 inhibition promotes a movement of total PKCε to the insoluble/membrane fraction** when the expected result would be the contrary. The literature and the rest of our data indicate that this PKCε pool becomes inactive after M_1 inhibition: M_1 is a $G_{q/11}$-linked receptor and all PKC substrates become less phosphorylated. Thus, it is possible that the inhibited PKCε becomes more associated with the cytoskeleton (Newton, 2010; Denning, 2012) or translocates between different

organelles (Shirai *et al.*, 1998), cell structures that are present in the analyzed insoluble fraction. On the other hand, PDK1 acts over cytoskeleton-associated PKC enzymes and releases them to the cytosol (Newton, 2010; Denning, 2012). Therefore, M_1 inhibition would decrease PDK1 activity and PKCε would be accumulated at the insoluble fraction (associated with the cytoskeleton). It is worth to mention that PKCε translocation seems not related to AKAP150, a PKA scaffold that also anchors PKC to the membrane (Navedo *et al.*, 2008; Zeng *et al.*, 2014; Perrino and Trimarco, 2017). This is because our results indicate that M_1 inhibition decreases AKAP150 levels in the insoluble fraction (Cilleros-Mañé *et al.*, 2020) in the conditions where PKCε is recruited into it.

A peculiar electrophysiological finding was that M_1 inhibition, supposed to inhibit PKC, actually induced the PKC coupling to neurotransmission (Santafé *et al.*, 2007). This could be related to our finding that PKCε translocates to the membrane after Pir. In particular, it is possible that PKCε at the membrane performs some activity, explaining the PKC coupling. On the other hand, both PKCβI and PKCε equally phosphorylate these substrates, which is probably due to the similarity in their kinase domain (Herget *et al.*, 1995). It is very interesting that both PKCβI and ε isoforms are necessary but do not replace each other ('necessary but not sufficient'). In coincidence, in electrophysiology, PKCε activity is a prerequisite necessary for classic PKC activity at the NMJ (Obis *et al.*, 2015b). This remarks the importance of studying the differences and cooperation between PKC isoforms.

Altogether, the above results provided precise molecular details over the potentiation of M_1 over ACh release at the NMJ. Overall, M_1 activates PKCβI and PKCε isoforms, induces their maturation through PDK1 and ultimately enhances the phosphorylation of some components of the vesicle release machinery.

1.2. M_1 crosstalk with the M_2/PKA pathway

Part of our experiments were dedicated to study the influence of M_1 over the M_2/PKA pathway. We expected interaction because both receptors are co-expressed in the presynapse of the NMJ (Garcia *et al.*, 2005) and PKA can be found in all the three cell components of the NMJ (Perkins *et al.*, 2001).

First, we found that **M_1 mAChR inhibition does not change M_2 protein levels** at the NMJ (■ Figure 17). In concordance, electrophysiology experiments demonstrate that the M_1 inhibition does not affect the outcome of M_2 inhibition (Santafé *et al.*, 2003, 2007). However, the M_1 mAChR could still affect the PKA pathway. First, PKC signaling modulates the expression of PKA subunits in cultured cells (Garrel *et al.*, 1993, 1995). Secondly, M_1 mAChR can link non-preferential G proteins (e.g. G_s and $G_{i/0}$) (Beguin *et al.*, 1996; Burford and Nahorski, 1996; Jakubík *et al.*, 2011) and, in hippocampal neurons, M_1 muscarinic signaling induces PKA activity (Zhao *et al.*, 2019).

We found that **M_1 signaling upregulates the PKA RIIβ subunit protein levels** (■ Figure 17). Elevated levels of R subunits are known to reduce PKA catalytic activity (Spaulding, 1993). The presynaptic M_1 receptors probably modulate postsynaptic RIIβ (Perkins *et al.*, 2001) through the upregulation of ACh release. Less evident, punctuated staining of M_1 labelling has also been observed at the postsynaptic folds (Malomouzh *et al.*, 2011), where it could also influence RIIβ.

Using atropine, we detected that some M_2-induced changes on the PKA pathway need non-blocked M_1 receptors. In particular, **M_1 activity is required by M_2 to decrease the Cβ protein levels and increase RIIα**. On the other hand, both M_1 and M_2 pathways increase RIIβ levels, which could cooperate to inhibit PKA activity. Atropine or Pir+Met treatments do not downregulate RIIβ more than Pir or Met alone. This inability to further inhibit the levels of RIIβ might be explained by mechanisms other than muscarinic receptors which could be buffering and protecting the synapse from an excessive decrease of the subunit. In this regard, our preliminary data indicates that exogenous BDNF (BDNF/TrkB pathway) increases RIIβ and prevents RIIβ levels from falling excessively (unpublished result).

Regarding subcellular location, **M_1 receptors constitutively recruit RIα, RIβ and RIIα to the membrane.** This is in opposition to the main pathway of M_2/PKA, which liberates RIβ and RIIα to the cytosol (see below). Since R and C get separated, the result suggests that M_1 downregulates the activity of catalytic subunits. On the other hand, we did not observe PKA translocation with atropine treatment. The absence of effect when both M_1 and M_2 are blocked could be because their opposed actions cancel each other out or, alternatively, because one subtype is necessary for the other. We speculate that the coexistence of both mAChR subtypes may balance the presence of RIβ and RIIα in the cytosol to finely control PKA C activity.

After finding that M_1 induced the translocation of PKA subunits, we evaluated if the protein scaffold AKAP150 was implied. Our results indicate that M_1 mAChR inhibition decreases the protein levels of AKAP150 at the membrane fraction. Thus, **M_1 mAChR increases AKAP150 and thus PKA anchoring sites** to the membrane, in line with its recruitment of R subunits to the membrane. Impaired Ca^{2+} signaling–which occurs after M_1 inhibition–has been linked to AKAP150 downregulation in a heart failure model (Li *et al.*, 2017). We tested PKC and PKA role over the expression of AKAP150 using blockers of PKC epsilon isoform (PKCε), beta I isoform (PKCβI) and PKA. All the three kinases promoted AKAP150 protein levels and were needed for the M_1-upregulation and the M_2-downregulation of AKAP150 (results not shown). It is stimulating to think that AKAPs provide a central platform to regulate and optimize multiple signaling pathways that converge at NMJs.

Our results show that **M_1 inhibition *per se* does not affect SNAP-25 Thr^{138} phosphorylation or CREB Ser^{133} phosphorylation at the NMJ.** It is surprising that although M_1 signaling modulates PKA, it does not modulate any of the substrates studied. We associate this with the **opposed actions of M_1 over the PKA pathway**: upregulating RIIβ protein levels (less PKA activity) and recruiting several R subunits to the membrane away from cytosolic catalytic subunits (more PKA activity).

In the following section we will review how only M_2 signaling modulates SNAP-25 Thr^{138} and CREB Ser^{133} phosphorylations. However, here it is noteworthy that **M_2 needs M_1 operativity**: blocking both subtypes

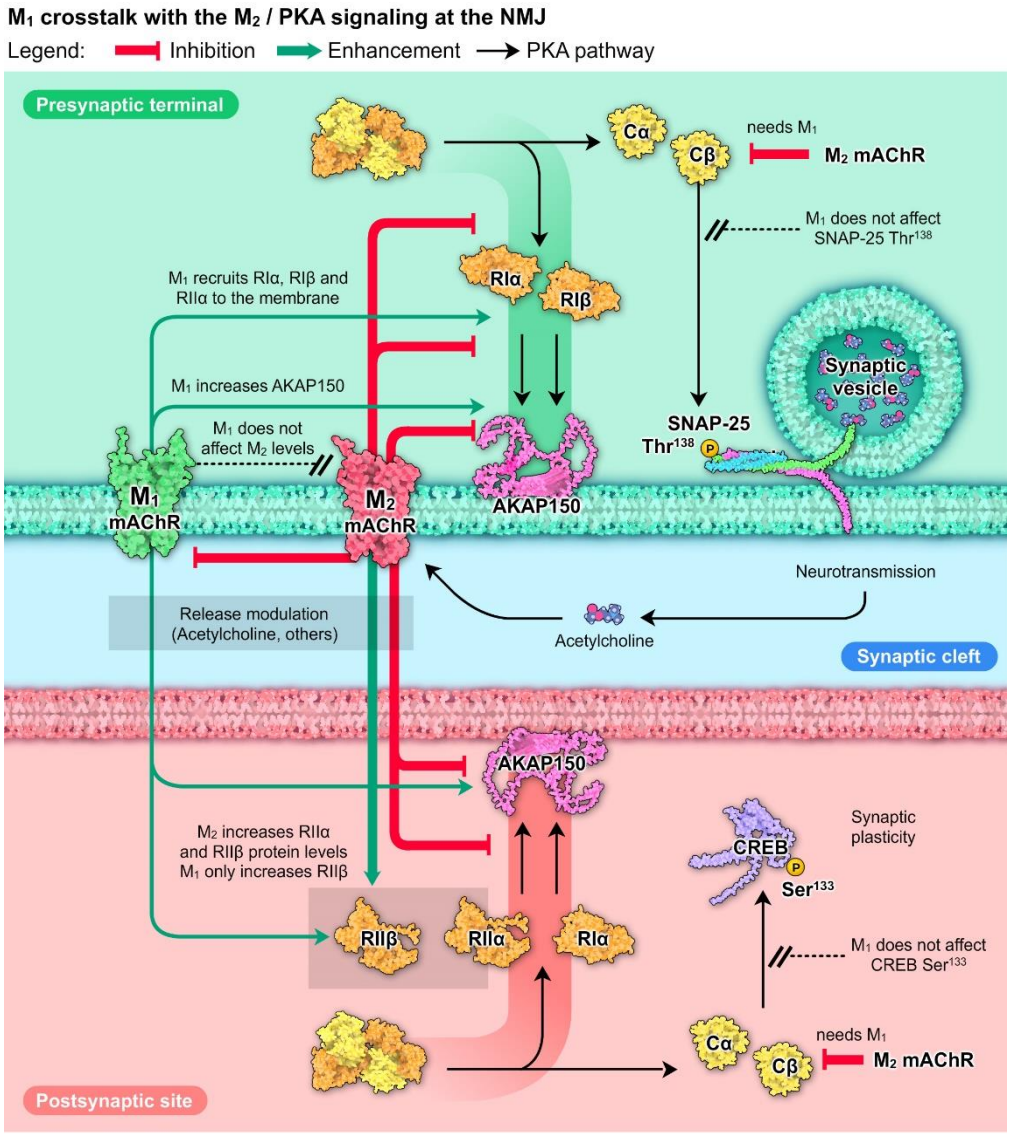

Figure 17. **M_1 crosstalk with the M_2/PKA pathway at the NMJ.** The M_1 signaling counterbalances many M_2 regulations over PKA subunits. M_1 receptors recruit RIα, RIβ and RIIα to the membrane, which might be related to the parallel enhancement of the scaffold AKAP150 levels. On the other hand, M_1 signaling upregulates the RIIβ subunit protein levels, mainly expressed at the postsynaptic site. M_1 inhibition alone does not affect SNAP-25 Thr^{138} or CREB Ser^{133} phosphorylation. However, M_2 needs M_1 operativity to decrease the Cβ protein levels and also to reduce the phosphorylation of both substrates. *Source: own elaboration.*

(with Atr or with a mixture of Pir+Met) prevents the M_2 decrease of SNAP-25 Thr^{138} and CREB Ser^{133} phosphorylation. This complex mechanism will benefit from research regarding (1) the dependency between PKC and PKA phosphorylation sites, (2) the actions of calcium signaling over cAMP levels (Choi *et al.*, 1992; Yan *et al.*, 1994) and (3) the PKC regulation of PKA subunit expression (Garrel *et al.*, 1993, 1995).

2. M_2 muscarinic signaling at the NMJ

The **M_2 muscarinic signaling inhibits neurotransmission** in cholinergic synapses, including the neuromuscular junction (Caulfield and Birdsall, 1998; Slutsky *et al.*, 1999; Santafé *et al.*, 2007). M_2 signaling occurs constitutively, as M_2 is active in basal conditions (Seifert and Wenzel-Seifert, 2002) and **PKA does not require external coupling factors** to potentiate neurotransmission at the NMJ (Santafé *et al.*, 2007). In this thesis, we have aimed to obtain molecular data about the M_2 signaling at the NMJ. In this section, we review our results about the modulation of M_2 over PKA, its target SNAP-25 Thr^{138}, the scaffold AKAP150 (Cilleros-Mañé *et al.*, 2020), and how M_2 also cross-modulates the M_1/PKC pathway(Cilleros-Mañé *et al.*, 2021).

2.1. The muscarinic M_2/PKA pathway at the NMJ

At the skeletal muscle, the inhibition of M_2 receptors enhances PKA and ACh release (Santafé *et al.*, 2003, 2007). The M_2/PKA pathway occurs near NMJs. This is because M_2 receptors are exclusively found at the presynaptic terminals (Garcia *et al.*, 2005; Wright *et al.*, 2009) and PKA subunits are localized close to synaptic regions (Perkins *et al.*, 2001). However, M_2 mAChRs affect neurotransmission and, thus, their action can modulate both presynaptic and postsynaptic PKA subunits. Therefore, we took special care with our interpretation of the results and **considered M_2 effects over the three cells of the NMJ** (■ Figure 18-A). In some experiments in this thesis, we sought to demonstrate specific presynaptic changes by studying the phosphorylation of SNAP-25, a substrate exclusive of nerve terminals (Simó *et al.*, 2019).

Since long time ago, PKA has been implicated in synaptic plasticity, enhancing the probability of release in the nerve terminal (Swope *et al.*, 1999; Nguyen and Woo, 2003; Taylor *et al.*, 2012) and controlling the postsynaptic response (Hoover *et al.*, 2002; Li *et al.*, 2002; Rudolf *et al.*, 2013). Some of these actions involve the phosphorylation of the release machinery and its regulatory molecules (reviewed in Leenders and Sheng, 2005).

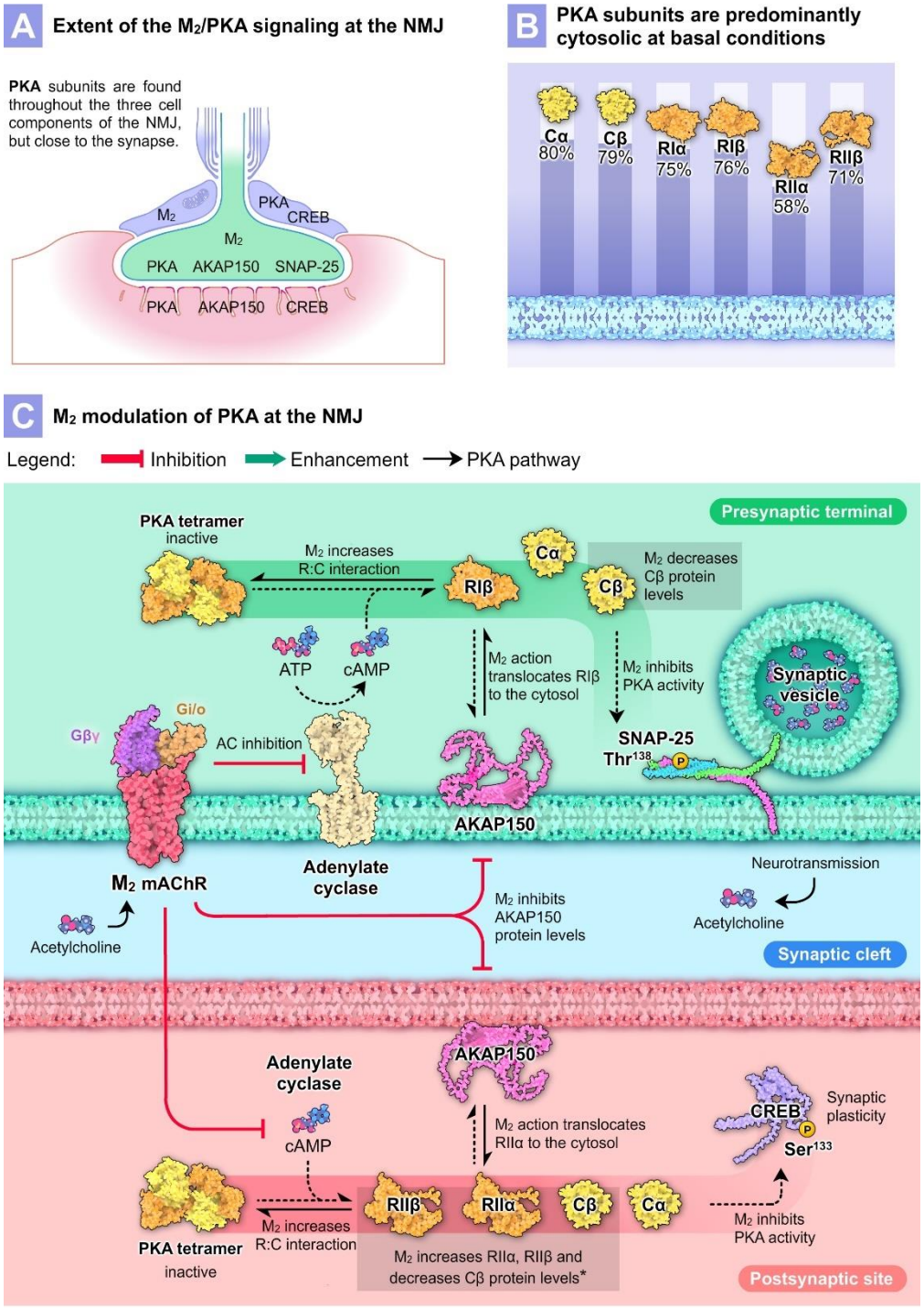

Figure 18. **M_2 muscarinic signaling over the PKA pathway at the NMJ.** A PKA kinase is ubiquitous and, thus, we considered both presynaptic and postsynaptic effects. We studied SNAP-25 to obtain data exclusive of nerve terminals. B Percentage of PKA subunits in the cytosolic fraction of the rat diaphragm at basal conditions. C M_2 mAChR blocks PKA activity by inhibiting adenylate cyclase (AC) and cAMP production. In this thesis we found that M_2 decreases the protein level of Cβ and increases RIIα and RIIβ, further reducing PKA activity. In addition, M_2 decreases AKAP150 levels and, in parallel, releases RIβ and RIIα to the cytosol where C subunits are abundant. *RIIα and RIIβ presynaptic locations should not be ruled out. M_2 also enhances the R:C interaction of RIβ with Cα and Cβ. Overall, M_2 decreases the phosphorylation level of the PKA substrates SNAP-25 (Thr^{138}) and CREB (Ser^{133}). *Source: own elaboration.*

PKA activity is regulated by changes in its protein level through synthesis and degradation (Hegde et al., 1993; Garrel *et al.*, 1995; Hoover *et al.*, 2002). We found out that the **M_2 mAChR signaling downregulates Cβ and upregulates RIIα and RIIβ**. This could be associated with a decrease in PKA action, since decreased C subunits and elevated R subunits downregulate catalytic activity (Spaulding, 1993). We also studied this regulation under phrenic nerve stimulation. In these conditions, synaptic activity increases Cβ levels and M_2 signaling still downregulates them, possibly to balance PKA activity. Unpublished results in our laboratory suggest that neurotrophic signaling might be responsible for increasing Cβ during synaptic activity (Polishchuk *et al.*, in preparation).

Next, we studied the PKA distribution between the membrane and cytosol, which affects its activity and substrate specificity (Reinitz *et al.*, 1997; Taylor *et al.*, 2012). At the rat diaphragm, **Cα, Cβ, RIα, RIβ and RIIβ subunits are mainly associated with the cytosol fraction** (~75% cytosol/total) and **only RIIα has less presence in the cytosol** (58%) (■ Figure 18-B). In consistence, PKA RI type is frequently diffused in the cytoplasm of cells, whereas RII type usually associates with membrane compartments (Brandon *et al.*, 1997; Reinitz *et al.*, 1997; Keryer *et al.*, 1999; Perkins *et al.*, 2001) due to their higher affinity to PKA-anchoring proteins (Wong and Scott, 2004). PKA activity is regulated by cytosol/membrane translocation of both C (Tillo *et al.*, 2017) and R subunits (Reinitz *et al.*, 1997; Stefan *et al.*, 2011). In the adult rat diaphragm, we observed that PKA R subunits are more prone to muscarinic-mediated translocation than C subunits. Our data indicate that **M_2 signaling liberates RIβ and RIIα to the cytosol**. RI subunits associate with the membrane fraction when they are not associated with C subunits (Reinitz *et al.*, 1997). Thus, we hypothesized that the RIβ and RIIα translocation mediated by M_2 signaling could be linked to changes in PKA R:C interaction. A similar mechanism was proposed by Stefan *et al.*, 2011, where a GPCR-mediated cAMP elevation promotes dissociation of the PKA heterotetramer and recruitment of R subunits to $G\alpha_{i/0}$ proteins at the membrane. We corroborated our finding with co-immunoprecipitation experiments,

showing that **M_2 inhibition strongly dissociates RIβ subunits from Cβ and Cα**, all of them **abundant in the synaptic region** of the skeletal muscle.

Even though M_2-mediated decrease in cAMP and the recruiting action of $G\alpha_{i/0}$ protein explained PKA translocation (Stefan *et al.*, 2011), this mechanism could not clarify the participation of M_1 signaling. Thus, we asked whether another factor was involved, namely a protein scaffold that targeted kinases to the membrane. The classical targeting of PKA to specific cell sites is largely achieved by AKAPs (Wong and Scott, 2004; Pidoux and Taskén, 2010), whose expression is modulated in several systems (Nomura *et al.*, 2002; Poppinga *et al.*, 2015; Li *et al.*, 2017). Our data shows that **M_2 mAChR inhibition increases the protein levels of AKAP150**. This might be a result of the potentiation of ACh release and neuromuscular activity by M_2 inhibition. In concordance, exercise training increases AKAP150 expression in rat fat tissue (Nomura *et al.*, 2002). Thus, muscarinic signaling appears to modulate AKAP150 levels and, doing so, the subcellular translocation of PKA R subunits.

The mechanism defined above implies less PKA activity, because the close association of regulatory and catalytic subunits prevents the phosphorylating activity of the catalytic ones (Taylor et al., 2012). We confirmed a decrease in PKA activity by checking the level of substrate phosphorylation. Accordingly, we found that **M_2 signaling inhibits the PKA-phosphorylation of SNAP-25 (Ser^{187}) and CREB (Ser^{133})**, which will be discussed in the next section. Altogether, our data provided some molecular clues about the predominance of M_2 signaling over M_1 at the NMJ. Overall, M_2 downregulates Cβ protein levels and enhances RIIα and RIIβ. On the other hand, M_2 also translocates of R subunits to the cytosol to increase the formation of PKA tetramers, which results in a reduced phosphorylation of SNAP-25 and CREB.

2.2. M_2 crosstalk with the M_1/PKC pathway

Previous studies at the NMJ predicted some influence of M_2 mAChR over the M_1/PKC pathway. First, because M_2 functionally dictates the muscarinic signaling at the NMJ and general muscarinic inhibition (atropine) produces effects more similar to M_2 inhibition than M_1 (Slutsky *et al.*, 1999; Santafé *et al.*, 2007). This was not simply due to a stronger M_2 activation: although M_2 has higher affinity for ACh than M_1 (Kellar *et al.*, 1985; Spencer *et al.*, 1986; Minic *et al.*, 2002), atropine has the same affinity for both mAChR subtypes (Caulfield and Birdsall, 1998). Secondly, because M_2 modulates PKA, which is a requisite for PKC activity at the NMJ (Santafé *et al.*, 2009). Indeed, the interrelation between M_2 and the M_1/PKC pathway is further proven by other studies showing that PKC is necessary for the M_2 muscarinic signaling in various neuromuscular models (Shen and Mitchelson, 1998; Wang *et al.*, 1999; Santafé *et al.*, 2007). Thus, some of our experiments were aimed to study whether the M_2 mAChR regulates molecules belonging to the M_1 pathway.

First, we found that **M_2 signaling downregulates the protein levels of M_1 receptors at the NMJ** (■ Figure 19). This provided an initial molecular explanation to why M_2 functionally overcomes M_1 in electrophysiology (Slutsky *et al.*, 1999; Santafé *et al.*, 2007). M_2 might decrease M_1 receptor by inhibiting PKA and its up-regulation of M_1 synthesis (Hao *et al.*, 2005) or, alternatively, affecting M_1 internalization and degradation (Scherer and Nathanson, 1990). The fact that M_2 downregulates M_1 protein levels and not vice versa is in line with different internalization mechanisms (Scherer and Nathanson, 1990). In particular, M_1 mAChR is internalized in a β-arrestin/dynamin dependent manner, whereas sequestration of M_2 seems largely independent of these proteins (Pals-Rylaarsdam *et al.*, 1997; Vögler *et al.*, 1999). Interestingly, we found that **M_2 needs M_1 activity to reduce the own M_1 levels**, because the effect is not observed when both receptors are blocked. M_1/PKC might be involved, as PKC promotes GPCR kinase (GRK) activity and β-arrestin/dynamin dependent internalization (Krasel *et al.*, 2001; Penela *et al.*, 2003). In line with all that, PMA treatment internalizes more M_1 receptors than M_2 and requires PKA presence (Scherer and Nathanson, 1990).

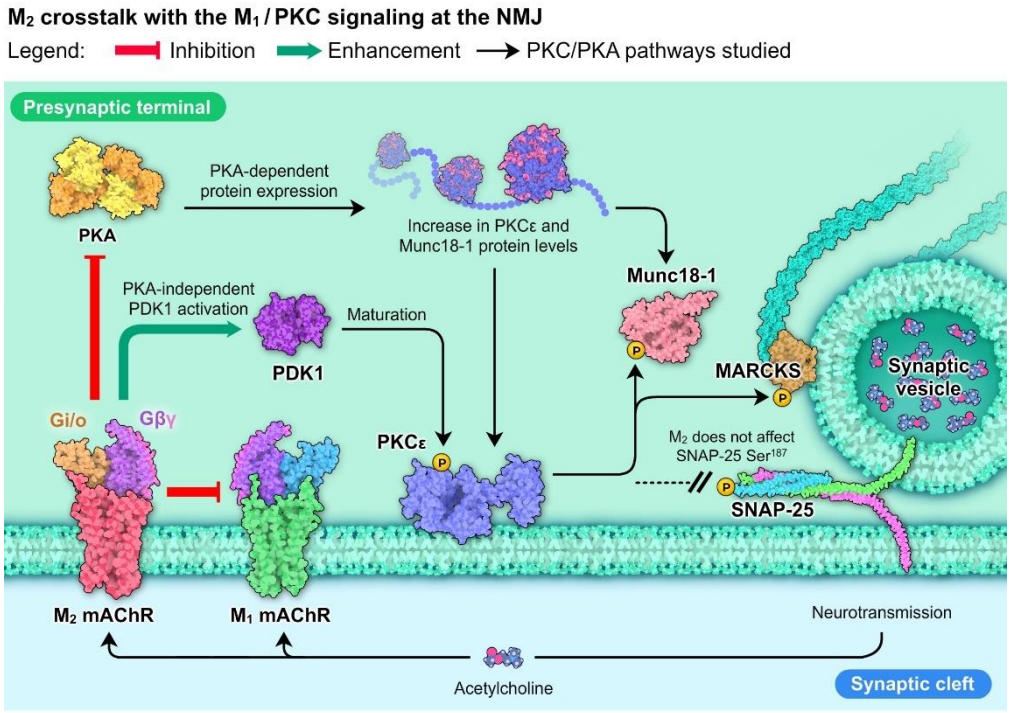

Figure 19. **M_2 crosstalk with the M_1/PKC pathway at the NMJ.** First, M_2 signaling interacts with the M_1/PKC pathway at the receptor level, decreasing the protein amount of M_1 receptors. Regarding kinases, two opposed pathways are depicted from M_2 signaling: one PKA-dependent and another PKA-independent. M_2 involves PKA to decrease the protein levels of PKCε and Munc18-1. In concordance with the PKCε decrease, the phosphorylation of the substrates Munc18-1 and MARCKS also decreases in a PKA- and PKCε-dependent manner. These downregulations occur at the membrane fraction and likely contribute to the decrease in neurotransmission (PKA-dependent pathway). On the other hand, when PKA is blocked, M_2 shifts to display actions similar to M_1 signaling, undercovering a PKA-independent pathway. In particular, M_2 enhances the PDK1 Ser^{241} phosphorylation, indicative of its activation. In turn, PDK1 promotes the maturation of PKCε in the membrane fraction. In this context without PKA, M_2 shifts to enhance the PKC-phosphorylation of both Munc18-1 and MARCKS (PKA-independent pathway). Neither PKCβI nor SNAP-25 Ser^{187} (PKC) phosphorylation are affected by M_2. *Source: own elaboration.*

Another clear evidence of M_2 influence over M_1/PKC is that M_2 inhibition couples PKC to neurotransmission (Santafé *et al.*, 2007). However, it was unknown if muscarinic receptors had different preference for particular PKC isoforms. In this thesis, we studied the classical PKCβI and the novel PKCε isoforms as presynaptic isoforms (Perkins *et al.*, 2001; Obis *et al.*, 2015a; Hurtado *et al.*, 2017a) that participate in synaptic transmission (Morgan *et al.*, 2005; Obis *et al.*, 2015b; Hurtado *et al.*, 2017a; Simó *et al.*, 2018, 2019). We did not find any link between M_2 signaling and the classical PKCβI isoform: **M_2 does not affect PKCβI priming, levels, subcellular distribution and this isoform**

is not required for any substrate phosphorylation affected by M_2 blockade that we studied at the NMJ (Cilleros-Mañé *et al.*, 2021). In concordance, M_2 has been observed to recruit novel instead of classical PKC isoforms in portal vein myocytes (Callaghan *et al.*, 2004). Indeed, we found that the novel PKCε isoform is affected by the M_2 pathway and required for many of its regulations at the NMJ. For example, **M_2 signaling promotes the maturation of PKCε at the membrane fraction** (■ Figure 19). In accordance, **M_2 also enhances the phosphorylation level of PDK1**, whose activity is responsible of PKCε priming (Cilleros-Mañé *et al.*, 2021). M_2 might activate PDK1 through the Gβγ subunit and a consequent PI3K activation (Wang *et al.*, 1999; Callaghan *et al.*, 2004). Because these changes are equal to the M_1 mAChR pathway, we proposed a **convergent branch** between M_1/M_2 over PDK1/PKCε priming. Accordingly, we showed that the inhibition of both M_1 and M_2 with atropine (Caulfield and Birdsall, 1998) also decreases the phosphorylation of PDK1. Interestingly, the atropine effect is not greater than the isolated M_1 or M_2 inhibitions. This lack of additive effect reinforces the idea that both receptors use the same pathway, specifically PI3K activation (Wang *et al.*, 1999; Callaghan *et al.*, 2004; Zhao *et al.*, 2019), which is linked to PDK1. Neuromuscular activity enhances PKC maturation at the NMJ (Hurtado *et al.*, 2017b) and, with this data, not only M_1 but also M_2 signaling would contribute to upregulate PKC priming. We are currently developing experiments in our laboratory to test this possibility (Polishchuk *et al.*, in preparation).

On the other hand, we found that **M_2 signaling downregulates PKCε protein levels in the membrane fraction** (■ Figure 19). As before, we considered all the available data to propose whether PKC downregulation was due to activity-dependent degradation (Lee *et al.*, 1996; Lu *et al.*, 1998; Kang *et al.*, 2000; Hurtado *et al.*, 2017a) or less PKC synthesis. In this case, evidence discarded a PKCε activation by M_2. First, M_2 inhibition activates and couples PKC at the NMJ (Santafé et al., 2007) and PKCε is not very sensitive to activity-dependent degradation (Olivier and Parker, 1992). On the other hand, less PKCε synthesis and activity were more reasonable, because **M_2 signaling uses PKA to decrease PKCε protein levels** and **M_2 also uses PKCε to decrease the phosphorylation of PKC substrates** (see below).

We also assessed the combined action M_1 and M_2 receptors over PKC levels with atropine (Caulfield and Birdsall, 1998). Atropine affects PKCε but not PKCβI, a similar effect to inhibiting only M_2 signaling. Therefore, **M_2 appears to have a greater overall effect over the PKC protein levels than M_1**. The upregulation of PKCε by atropine can be explained by the sum of inhibitions of M_1 and M_2. On the one hand, atropine blocks M_1 thus decreasing PKCε activity. However, the accompanying M_2 blockade by atropine liberates PKA activity, which upregulates PKCε levels and buffers the action of M_1. It is also possible that, because atropine does not replicate the effect of M_1 inhibition over PKCβI protein levels, M_1 might need an active M_2 to modulate PKCβI turnover. In particular, we demonstrated lack of other mAChR subtype influence by replicating these findings with a mixture of Pir+Met. Overall, the M_1/M_2 combined action probably balances PKCε activity and protects the synapse from an excessive or insufficient PKC activity.

Finally, we determined PDK1 activity over PKC substrates. Overall, PDK1 blockade has similar effects to M_1 inhibition, regulating in an equal manner PKCβI, PKCε and the substrates SNAP-25 and MARCKS. This supports that M_1 signaling relies mainly on the PKC pathway, where PDK1 plays an important role. On the other hand, M_2 blockade also inhibits PDK1 activity and we observed that PDK1 blockade *per se* induces effects similar to M_2 inhibition over Munc18-1 phosphorylation. Interestingly, atropine does not induce the same modulations as PDK1 inhibition, although we determined PDK1 as a common M_1/M_2 node. This is probably because PDK1 inhibition only disrupts PKC priming, one step of PKC activation, whereas atropine induces a pan-muscarinic inhibition, involving both the PKC pathway–including PDK1–and the PKA pathway.

M_2 inhibition activates and couples PKC to participate in acetylcholine release at the NMJ (Santafé et al., 2007). We observed that **M_2 inhibition induces PKC substrate phosphorylation**, upregulating Munc18-1 Ser^{313} PKC-phosphorylation, Munc18-1 protein levels and MARCKS $Ser^{152/156}$ PKC-phosphorylation. These **M_2 modulations over PKC isoforms,**

Munc18-1 and SNAP-25 occur in the synaptic region of the hemidiaphragm. Only Munc18-1, which is also present in the nerve axons and branches (Simó *et al.*, 2018), also showed a tendency increase outside the synaptic region. The responsible PKC isoform is likely PKCε, as it upregulates both Munc18-1 phosphorylation and protein levels (Simó et al., 2018) and phosphorylates MARCKS (Obis *et al.*, 2015a). Also, here we verified with double-inhibitor experiments that **PKCε activity is required for M_2 blockade to upregulate Munc18-1 and MARCKS**. Thus, the increase in PKCε by M_2 inhibition is consistent with enhanced PKC activity and it is possibly caused through protein synthesis (see below for the implication of PKA).

We next tested whether PKA activity was the link between M_2 and PKCε. Our results show that M_2 blockade involves PKA activity to increase PKCε levels but does not need PKA to decrease PKCε phosphorylation. This reveals that M_2 signaling affects PKCε through two different pathways: a **PKA-dependent pathway** where M_2 downregulates PKCε, and a **PKA-independent pathway** where M_2 induces phospho-PDK1 and PKCε maturation. The mentioned PKA-independent pathway is concordant with the previous studies linking M_2 and novel PKC through the Gβγ subunit and PI3K activation (Wang *et al.*, 1999; Callaghan *et al.*, 2004), hence not needing G_i protein or PKA activity. On the other hand, M_2 reduces PKCε levels via G_i protein and PKA inhibition. In other words, when M_2 is blocked, PKA activity increases and upregulates PKCε. In this condition, phospho-PKCε does not increase along the total levels because PDK1 remains downregulated by Met.

We believe that the M_2 upregulation of PKCε might involve a **posttranscriptional mechanism** because PKCε is exclusively located at the synaptic terminal of NMJs (Obis *et al.*, 2015b), where gene transcription does not take place. PKA activity has been linked to the modulation of mRNA translation through the phosphorylation of the eukaryotic elongation factor 2 kinase, inducing a general reduction of mRNA translation but increasing the translation rate of a small subset of synaptic proteins (Chotiner *et al.*, 2003; Heise *et al.*, 2014). Also, PKA promotes protein translation via the

phosphorylation of polypyrimidine tract-binding protein 1, a molecule that participates in pre-mRNA splicing (Knoch *et al.*, 2006).

Additionally, we also checked the effect of M_2 over PKCα, a ubiquitous isoform mainly expressed at the postsynaptic site of the NMJ. **M_2 mAChR downregulates the levels and priming of PKCα.** This result is another indicator that M_2 mAChR signaling spans over postsynaptic proteins, likely due to its actions over ACh release. In this case, our data suggests that M_2 signaling on PKCα could be linked to a reduction of the postsynaptic responsiveness to ACh.

It is remarkable that M_2 modulates SNAP-25 Thr^{138} phosphorylation (PKA) at the NMJ (Cilleros-Mañé *et al.*, 2020) but not SNAP-25 Ser^{187} (PKC) phosphorylation. We demonstrated that M_2 requires PKA activity to modulate Munc18-1 and MARCKS. Interestingly, we found that all PKC phosphorylations inhibited by M_2, and hence attributed to PKCε, require PKA activity. Taken together, our data suggests that **M_2/PKA action occur upstream and control PKCε levels and activity,** complimenting accordingly the electrophysiology results demonstrating the PKCε dependency of M_2 (Obis *et al.*, 2015b).

Interestingly, we found that **a previous inhibition of PKA shifts M_2 to perform an M_1-like signaling** in relation to Munc18-1 and MARCKS phosphorylation. In other words, after H-89 preincubation, M_2 blockade decreases both Munc18-1 and MARCKS phosphorylation, which is the result of M_1 inhibition. This is concordant with previous functional studies showing that when PKA is previously inactivated, blocking either M_1 or M_2 leads to a similar reduction in transmitter release (Santafé *et al.*, 2007). Indeed, this experiment appears to **unmask the PKA-independent pathway** described before where M_2 induces PDK1 and PKCε phosphorylation. Thus, without PKA activity in the system, M_2 blockade would just downregulate PDK1 and PKCε phosphorylations (our results) through Gβγ/PI3K (Wang *et al.*, 1999; Callaghan *et al.*, 2004), causing a decrease in Munc18-1 and MARCKS PKC-phosphorylation.

Furthermore, we show that **M_1 signaling does not require PKA to promote the PKC phosphorylation of Munc18-1 (Ser^{313}) or MARCKS**

(**Ser$^{152/156}$**). However, PKA affects the protein levels of MARCKS. This is because, without PKA activity, M_1 blockade reduces MARCKS levels. As it will be further discussed, this might be caused by the role of PKA in protein translation (Chotiner *et al.*, 2003; Knoch *et al.*, 2006; Heise *et al.*, 2014). **In contrast, M_1 does require PKA for the PKC phosphorylation of SNAP-25 (Ser^{187}).** PKA activity *per se* phosphorylates SNAP-25 over Thr^{138} (Hepp *et al.*, 2002; Leenders and Sheng, 2005) which also occurs at the NMJ (Cilleros-Mañé *et al.*, 2020). Therefore, PKA phosphorylation of SNAP-25 Thr^{138} might be required for the phosphorylation on Ser^{187}. Alternatively, the PKA influence over SNAP-25 Ser^{187} might be due to the described modulation of PKCε.

Therefore, M_2 influences the M_1/PKC pathway besides its main pathway decreasing PKA activity by modulating the protein levels and translocation of PKA subunits. At the receptor level, M_2 downregulates M_1 protein levels. In a PKA-independent manner, M_2 promotes PDK1 activity and PKCε maturation. However, the PKA-dependent pathway inhibits PKCε protein levels, Munc18-1 protein levels, SNAP-25 Ser^{138} phosphorylation and is required for SNAP-25 Ser^{187} (PKC) phosphorylation. Altogether, these results provide a molecular explanation about the predominance of M_2 signaling over M_1 at the NMJ.

3. Overall muscarinic modulation at the NMJ

3.1. Phosphorylation of the release machinery

We completed our study of the M_1/PKC and M_2/PKA pathways by characterizing the phosphorylation of **SNAP-25** (Genoud *et al.*, 1999; Risinger and Bennett, 1999; Hepp *et al.*, 2002), **Munc18-1** (Leenders and Sheng, 2005; Snyder *et al.*, 2006; Simó *et al.*, 2018), **CREB** (Kaplan *et al.*, 2017) and **MARCKS** (Yang *et al.*, 2002). With that, we also tried to link the phosphorylation of these particular proteins to the physiological regulation of exocytosis by muscarinic signaling. In the following sections, we discuss our findings and propose the role of these data over neurotransmission.

It should also be noted that the synaptic release machinery is extraordinarily complex and PKA and PKC have a myriad of other targets with immediate or long-term roles. To mention just a few: the Cysteine String Protein (CSP) (Evans et al., 2001), N-ethylmaleimide-sensitive factor Attachment Protein alpha (α-SNAP) (Hirling and Scheller, 1996), Rab3 Interacting Protein 1α (RIM1α) (Lonart *et al.*, 2003), rabphilin (Lonart and Südhof, 1998), Snapin (Chheda et al., 2001), synapsin I (Jovanovic *et al.*, 2001) and syntaphilin (Boczan *et al.*, 2004).

SNAP-25

SNAP-25 is a SNARE core protein of the fusion vesicle complex involved in vesicle docking, priming and triggering fast exocytosis (Söllner *et al.*, 1993b; Mohrmann *et al.*, 2010). In this thesis we have studied two SNAP-25 phosphorylations. The **PKA phosphorylation** of SNAP-25 at Thr^{138} controls the size of the releasable vesicle pools, but does not participate in the assembly and stability of ternary SNARE complex (Risinger and Bennett, 1999). In this thesis, we report that Thr^{138} at the NMJ can be decreased with the PKA inhibitor H-89 (Cilleros-Mañé *et al.*, 2020). In contrast, the **PKC phosphorylation** at Ser^{187} regulates the SNARE complex formation (Kataoka *et al.*, 2000; Shu *et al.*, 2008; Katayama *et al.*, 2017) and the refilling after the pools have been emptied (Nagy *et al.*, 2002; Leenders and Sheng, 2005). At the NMJ, SNAP-25 Ser^{187} can be decreased with low external calcium (Simó *et al.*, 2019).

Regarding the M_1 pathway, we showed that **M_1 increases the SNAP-25 phosphorylation of Ser^{187} (PKC) but not Thr^{138} (PKA).** The PKC phosphorylation by M_1 occurs at the membrane fraction, in accordance with its activation (de Vries *et al.*, 2000; Kataoka *et al.*, 2000; Disatnik *et al.*, 2004). These results fit properly with the known M_1 enhancement of ACh release at the NMJ and the coupling of PKC at the NMJ (Slutsky *et al.*, 1999; Santafé *et al.*, 2003). The absence of M_1 action over the PKA/SNAP-25 phosphorylation might be due to its opposed actions over the PKA pathway: recruiting the inhibitory R subunits (RIβ, RIIα, RIIβ) to the membrane –away from catalytic subunits– but at the same time increasing the levels of another R subunit (RIIβ). Currently, we are studying if conditions that modulate R subunit expression–like synaptic activity–can change the R subunit balance and undercover an M_1/SNAP-25 Thr^{138} regulation (Polishchuk *et al.*, in preparation). On the other hand, **M_1 mAChR uses both PKCβI and PKCε to phosphorylate SNAP-25 Ser^{187} at the NMJ**. This finding is in contrast with the earlier report that SNAP-25 Ser^{187} is phosphorylated by PKCε but not PKCβI during synaptic stimulus (Simó *et al.*, 2019). However, this discrepancy might be explained because M_1 inhibition and synaptic stimulus are different PKC coupling factors. All things considered, it is plausible that PKCβI and PKCε are complexly connected to SNAP-25, because both isoforms promote the neurotransmission and are influenced by many signaling pathways at the NMJ (Obis et al., 2015b, 2015a; Hurtado et al., 2017a). Surprisingly, **M_1 also requires PKA to promote SNAP-25 Ser^{187} phosphorylation.** This is the only PKC phosphorylation that we studied that needs PKA activity. Blocking PKA activity reduces phospho Thr^{138} levels, and this might influence the ability of M_1 to enhance Ser^{187} phosphorylation.

PDK1 activity promotes SNAP-25 (PKC) phosphorylation. This is similar to M_1 modulation and it is likely mediated by PKCβI and PKCε, whose priming is activated by PDK1. This result highlights the influence of PDK1 over PKC at the NMJ, although it is a master kinase that controls many others (Toker, 2003; Mora *et al.*, 2004; Bayascas, 2010; Pearce *et al.*, 2010). Unfortunately, PDK1 influence over NMJ electrophysiology is not known and it would be interesting to assess in the future.

Regarding the M_2 pathway, we determined that **M_2 reduces SNAP-25 phosphorylation of Thr^{138} (PKA) but not Ser^{187} (PKC)**. The PKA phosphorylation could be prevented with a previous incubation of H-89, demonstrating that M_2 inhibition **involves PKA**. The studied PKA Cβ subunit is highly likely to be involved, as it is expressed in the nervous system (Guthrie *et al.*, 1997; Enns *et al.*, 2009), and we located it within the synaptic region in all three cells of the NMJ. In addition, the Cβ interaction with RIβ subunits is controlled by M_2 signaling. SNAP-25 phosphorylation by PKA is necessary to maintain the ready-to-release and primed pools of vesicles. Thus, the M_2 inhibition of SNAP-25 Thr^{138} phosphorylation likely participates in the downregulation of ACh release (Slutsky *et al.*, 1999; Santafé *et al.*, 2007).

Finally, we used atropine to test the overall effect of muscarinic signaling. We found that **atropine does not modulate SNAP-25 PKC (Ser^{187}) nor PKA (Thr^{138}) phosphorylations at the NMJ** (Cilleros-Mañé et al., 2020, 2021). To our experience, SNAP-25 appears difficult to modulate with treatments, responding to neuromuscular activity and PKCε inhibition but not to PKCβI *per se* or TrkB inhibitions (Simó *et al.*, 2019). This might be due to the multiple mechanisms regulating it (Sørensen *et al.*, 2003; Leenders and Sheng, 2005; Simó *et al.*, 2019; Cilleros-Mañé *et al.*, 2020). Interestingly, atropine could not replicate the effect of M_1 inhibition nor the effect of M_2 inhibition (Cilleros-Mañé et al., 2020, 2021). This suggests that each muscarinic receptor requires the activity of the other to affect SNAP-25. If the inhibitory M_2 signaling on PKA/SNAP-25 is necessary for the M_1/PKC phosphorylation of SNAP-25, the current results could suggest that elevated PKA activity hinders the phosphorylation of PKC over SNAP-25. Further research will be needed to clarify this interesting yet complex interplay between PKC and PKA over SNAP-25. As a final remark, atropine's upregulation of quantal acetylcholine release (Slutsky *et al.*, 1999; Santafé *et al.*, 2007) could be explained by other PKC/PKA substrates. This includes, Munc18-1 (Leenders and Sheng, 2005; Snyder *et al.*, 2006; Simó *et al.*, 2018)–discussed in the following section–, Synaptotagmin (de Jong *et al.*, 2016), N-ethylmaleimide-sensitive factor (NSF) (Matveeva *et al.*, 2001; Pontier *et al.*, 2006; Chou *et al.*, 2010), and N-type calcium channels, voltage-gated Na^+ channels (Barrett and Rittenhouse, 2000).

Munc18-1

Munc18-1 is an SM protein absolutely essential for exocytosis (Verhage *et al.*, 2000). In this thesis, we studied Munc18-1 Ser^{313} PKC phosphorylation, which controls the association with Syntaxin-1, the SNARE pin formation and membrane fusion (Yamaguchi *et al.*, 2002; Südhof and Rothman, 2009). The PKC phosphorylation site Ser^{313} is not conserved in other SM proteins that are involved in different intracellular membrane fusion processes, suggesting that Ser^{313} plays some specific role in exocytosis modulation (Barclay *et al.*, 2003). In the following lines, it should be noted that Ser^{313} alone does not explain all Munc18-1 kinetics *per se* and works together with the PKC-phosphorylation over Ser^{306} to modify exocytosis (Fujita *et al.*, 1996; Barclay *et al.*, 2003).

We found that **M_1 mAChR signaling upregulates Munc18-1 Ser^{313} phosphorylation** without affecting its protein level at the NMJ. The PKC-phosphorylations of Munc18-1 (Ser^{306} and Ser^{313}) decrease Syntaxin-1 binding (Fujita *et al.*, 1996), allow faster release kinetics and a more rapid vesicle cycling (Barclay *et al.*, 2003). M_1 enhances phospho-Munc18-1 at the particulate fraction (membrane) and also induces the translocation of Munc18-1 from the cytosol to the membrane. Concordantly, Munc18-1 activation is closely related to its membrane association (de Vries *et al.*, 2000). The phospho Munc18-1 increase by M_1 is a piece of the puzzle that fits correctly in the well-known M_1 increase of EPP amplitude and coupling of PKC activity (Santafé *et al.*, 2007). As for the role of kinases, we observed that **M_1 muscarinic signaling requires PKCβI and PKCε but not PKA to phosphorylate Munc18-1 Ser^{313}**. This is in concordance with the classic M_1/PKC signaling (Caulfield and Birdsall, 1998; Slutsky *et al.*, 1999) and with our report that PKCε and PKCβI phosphorylate Munc18-1 under synaptic activity at the NMJ (Simó *et al.*, 2018).

On the other hand, **M_2 pathway downregulates both Munc18-1 protein levels and, hence, its Ser^{313} phosphorylation.** This result fits with the inhibitory role of M_2 receptors (Santafé *et al.*, 2007) for two reasons. First, lower levels of Munc18-1 PKC-phosphorylation would imply a decrease in

the kinetics of the synaptic release (Barclay *et al.*, 2003). Secondly, the lower protein amount of Munc18-1 is associated with less synaptic strength, as synapses that recruit less Munc18-1 after stimulation also have a smaller releasable vesicle pool (Cijsouw *et al.*, 2014). To understand what could cause the protein increase of Munc18-1 after M_2 inhibition, it is important to consider its exclusive nerve terminal localization at the NMJ (Simó *et al.*, 2018). Nerve terminals of motoneurons are far away from the nucleus and thus protein upregulation is achieved with local expression of mRNA pools (Giuditta *et al.*, 2008). Indeed, we showed that **M_2 involves PKA and PKCε to downregulate Munc18-1 at the NMJ, but not PKCβI.** As mentioned above, PKA promotes the translation of various proteins by phosphorylating the polypyrimidine tract-binding protein 1 (Knoch *et al.*, 2006) and increases the translation rate of a small subset of synaptic proteins by phosphorylating the eukaryotic elongation factor 2 kinase (Chotiner *et al.*, 2003; Heise *et al.*, 2014).

Contrarily to the other studied PKC substrates, **Munc18-1 Ser^{313} phosphorylation is downregulated by PDK1 activity.** PDK1 kinase is at the crossroads of M_1 and M_2 opposed pathways. In this case, PDK1 activity results in a similar regulation as M_2 signaling over Munc18-1 phosphorylation.

Next, we studied how atropine modulated the PKC activity. **Atropine downregulates Munc18-1 phosphorylation and increases its protein levels.** In comparison with the selective inhibitions, it appears that M_1 blockade is responsible for the decrease in phospho Munc18-1, whereas M_2 blockade is responsible for the increase in Munc18-1 protein levels. Therefore, the activities of both mAChRs balance each other: M_1 promotes Munc18-1 phosphorylation, via PKCβI and PKCε, whereas M_2 signaling controls the levels of this regulatory synaptic protein, via PKCε and PKA. The upregulation of Munc18-1 could be involved in the atropine's upregulation of ACh release at the NMJ (Slutsky *et al.*, 1999; Santafé *et al.*, 2006).

Finally, in a previous study we determined that synaptic stimulus elevates both Munc18-1 protein levels and Ser^{313} phosphorylation (Simó *et al.*, 2018). At the time, we did not know which receptor mediated that upregulation. Indeed, the BDNF/TrkB pathway, potentially through the

truncated TrkB T1 isoform, reduced Munc18-1 phosphorylation (Hurtado *et al.*, 2017a; Simó *et al.*, 2018). The present data shows that M_1 mAChR increases phospho-Munc18-1 and that M_2 inhibition increases its levels. Thus, M_1/M_2 signaling and ratio are a good candidate to explain the Munc18-1 changes during synaptic activity. At this time, experiments are being performed to test this hypothesis.

CREB

We analyzed **CREB** as a well-known PKA substrate. CREB is a long-lasting master regulator of gene expression that is modulated by various pathways, for example cAMP/PKA, ERK1/2, and PI3K/Akt (reviewed by Leenders and Sheng, 2005; Snyder *et al.*, 2006). We analyzed the phosphorylation on Ser^{133} for the PKA/CREB signaling pathway.

At the NMJ, we showed that M_2 inhibition at basal conditions upregulates CREB Ser^{133} phosphorylation. On the contrary, this phosphorylation can be decreased with a preincubation of the PKA inhibitor H-89. After a PKA blockade, a subsequent M_2 inhibition cannot re-phosphorylate this substrate. This indicated that **M_2 involves PKA to downregulate CREB Ser^{133} phosphorylation.** This is coincident with the described M_2-decrease of Cβ levels, increase of RIIα and RIIβ, the translocation of RIβ and RIIα to the cytosol and the final formation of the inhibited PKA tetramer. As for the location, this pathway occurs most likely in nucleated cells, mainly the postsynaptic myocytes. In particular, CREB expression and phosphorylation at the skeletal muscle occurs in the nuclei of myocytes and can be observed in both synaptic and extra-synaptic regions of fast- and slow-twitch muscles (Stewart *et al.*, 2011; Choi *et al.*, 2013). On the other hand, PKA subunits are expressed within NMJ vicinities but present in the three cell components (Perkins *et al.*, 2001).

Regarding the M_1 pathway, we found at first that M_1 blockade does not affect CREB phosphorylation at the NMJ. However, M_1 is still necessary, because **M_1/M_2 inhibition with atropine cannot replicate the effect of M_2 inhibition over CREB.** This meant that M_2 mAChRs need some M_1

operativity to modulate CREB. This result, similar to SNAP-25 (PKA) phosphorylation, could result from the many M_1-mediated modulations of PKA subunits and, in any case, supports the crosstalk between both mAChRs.

Altogether, the M_2 modulation of CREB at the NMJ suggests changes in gene expression that could modulate synaptic plasticity at the NMJ, as it does in the central nervous system (Kaplan *et al.*, 2017). It is promising to think that M_2 receptors could participate in the alterations of postsynaptic PKA during age and disease (Rudolf *et al.*, 2013; Just-Borràs *et al.*, 2019a). Our team is currently testing this question under the I+D+i project "Effect of neuromuscular activity on the interaction between the muscarinic and neurotrophic synaptic signaling. Role in Amyotrophic Lateral Sclerosis (MAChRALS)" funded by the Spanish *Ministerio de Ciencia, Innovación y Universidades*.

MARCKS

The fourth studied substrate was **MARCKS** and its PKC $Ser^{152/156}$ phosphorylation. MARCKS is required in the trafficking of vesicles along the neurites (Yang *et al.*, 2002) due to its activity rearranging the cytoskeleton and interacting with membranes (Vaughan *et al.*, 1998).

M_1 promotion of PKC phosphorylation over MARCKS has been reported (Willets *et al.*, 2007). Multiple PKC isoforms can phosphorylate MARCKS *in vitro* and *in vivo* (Herget *et al.*, 1995; Obis *et al.*, 2015a). Here, we determine that, at the NMJ, **M_1 recruits the presynaptic PKCβI and PKCε to phosphorylate MARCKS** at the membrane fraction. In line with that, **PDK1 activity alone also promotes MARCKS phosphorylation.** Although M_1 requires no PKA activity to phosphorylate MARCKS, PKA maintains the protein levels of the substrate. This is because, without PKA activity, M_1 blockade decreases MARCKS levels. PKA is involved in gene expression and protein translation (Chotiner *et al.*, 2003; Knoch *et al.*, 2006; Heise *et al.*, 2014), so shutting down PKA from the system could be revealing a MARCKS-degrading pathway induced by M_1 mAChR inhibition.

In opposition, **M_2 downregulates MARCKS PKC phosphorylation.** The PKC isoform responsible of that is **PKCε**, whose blockade abolishes the effect of M_2 over MARCKS. This finding is consistent with the reported interaction between M_2 and PKCε function at the NMJ (Obis *et al.*, 2015b) as well as the general coupling of PKC by M_2 unbalance (Santafé *et al.*, 2007). Using H-89 before M_2 blockade provided a very interesting result: **M_2 upregulates MARCKS PKC phosphorylation in absence of PKA activity.** Indeed, this result unmasks the PKA-independent pathway described before, where M_2 induces PKCε maturation and activity. Thus, once PKA is isolated, the effect of M_2 blockade is to downregulate PDK1, PKCε maturation and ultimately substrate phosphorylation. Remarkably, this finding provides a molecular clue to the electrophysiology data showing that when PKA is previously inactivated, M_2 inhibition shifts to decrease the transmitter release at the NMJ mimicking M_1 (Santafé *et al.*, 2007).

Regarding the general muscarinic signaling, **atropine did not affect MARCKS $Ser^{125/156}$ phosphorylation.** This is likely caused by the counter regulation between the effect of M_1 blockade (decrease) and the effect of M_2 blockade (increase), both of them using PKCε. MARCKS phosphorylation has been linked with PKCε (Obis *et al.*, 2015a). Concordantly, here we demonstrated that both mAChR subtypes modulate and require PKCε activity to adjust MARCKS phosphorylation.

Altogether, the opposed regulation of M_1 and M_2 mAChRs over MARCKS may be used to balance the size of the primed synaptic vesicle pool and ready-to-release pool at the NMJ, thanks to its role in vesicle trafficking (Yang *et al.*, 2002) and cytoskeleton–membrane interaction (Vaughan *et al.*, 1998).

3.2. Comment on atropine and the evaluation of the overall muscarinic signaling

In this thesis we used atropine to test the overall muscarinic signaling. As a pan-muscarinic inhibitor, atropine binds to all mAChR subtypes. Classic studies at the adult NMJ show that mainly M_1 and M_2 orchestrate the NMJ neurotransmitter release (Slutsky *et al.*, 1999; Minic *et al.*, 2002; Santafé *et al.*, 2004;

Wright *et al.*, 2009), whereas other mAChR subtypes like M_3 and M_4 only participate during the development of the newborn NMJ (Santafé *et al.*, 2004; Nadal *et al.*, 2016, 2017). To discard M_3-M_5 side effects, we compared the effects of atropine with the effect of a mixture of Pir+Met, which inhibit M_1 and M_2. This technique has been used in the frog NMJ, where the mixture fully imitates the effects of atropine on neurotransmitter release (Slutsky *et al.*, 1999) and, in rat hippocampal place cells, where the mixture replicates the effects of scopolamine, another known pan-muscarinic inhibitor (Brazhnik, 2004). We observed that the Pir+Met treatment and atropine caused the same effects in all the molecules observed, reinforcing the major participation of M_1 and M_2 signaling at the NMJ. However, we recognize that discriminating between muscarinic receptor subtypes is still an important limitation. The development novel mAChR orthosteric agonists (Bock *et al.*, 2012) and innovative techniques like muscarinic photoswitchable modulators (Riefolo *et al.*, 2019) would greatly overcome the limitation of muscarinic subtype selectivity.

4. Significance statement: the importance of the NMJ in health and disease

Much of this thesis is devoted to describing in detail the molecular cascade induced by the muscarinic signaling at the adult NMJ. As basic science, this research cannot promise to solve a specific health goal. However, as part of competitive government-funded projects (SAF2015-67143-P; PID2019-106332G-B-I00), it is very important for us to return what society has granted to us and consider the potential applicability of our work. This provides social value to our research and helps to identify interesting and unknown gaps in our knowledge to set out the path of our future research.

The study of NMJs as a synaptic model is useful as it is one of the synapses most susceptible to disease (Rodríguez Cruz *et al.*, 2020). In the first place, it is the target of many neurotoxins from bacteria, fungi, snakes and other species that disturb the neuromuscular transmission and cause potentially fatal complications. The study of these toxic factors has led to the

discovery of many pharmacological tools to study and modulate the neuromuscular activity, including the muscarinic drugs (Brown *et al.*, 1977; Miledi *et al.*, 1978; Robitaille *et al.*, 1990; Caulfield and Birdsall, 1998).

On the other hand, the NMJ can also be damaged by important autoimmune, genetic and degenerative disorders. Two autoimmune disorders of clinical importance are myasthenia gravis and the Lambert-Eaton myasthenic syndrome. Myasthenia gravis is caused by autoantibodies against nAChRs, and is classically treated with immunosuppressive drugs and cholinesterase inhibitors to prevent the breakdown of ACh by AChE (Rodríguez Cruz *et al.*, 2020). The Lambert-Eaton myasthenic syndrome is rarer and is mainly caused by autoantibodies against P/Q-type VGCCs and synaptotagmin 1 from the presynaptic terminal of NMJs (Eaton and Lambert, 1957). Although these are the mainly referred targets, autoantibodies against the M_1 mAChR are also implicated in this presynaptic disorder (Takamori, 2019). Interestingly, in a study of 25 Lambert-Eaton myasthenic syndrome patients, 19 patients (76 %) were positive for presynaptic M_1- type mAChR antibodies, and 4 of whom were negative for antibodies to both P/Q-type VGCC and synaptotagmin 1 (Takamori, 2008, 2019). Thus, the study of muscarinic receptor function, location and its signaling might become important to understand their role in nerve-muscle homeostasis, in dystrophy and myasthenia (Röder *et al.*, 2009; Rudolf *et al.*, 2013) and NMJ regeneration (Röder *et al.*, 2012).

On the other hand, the genetic alterations that affect NMJs are grouped in a family of heterologous disorders called congenital myasthenic syndromes (Beeson, 2016; Rodríguez Cruz *et al.*, 2020). Some examples that fall close to the research of our laboratory are mutations in the genes encoding for the transmission molecules SNAP-25 isoform B (SNAP25B), Munc13, synaptotagmin, sodium channels and the cholinergic molecules nAChR, AChE and choline acetyltransferase (ChAT) (Beeson, 2016). Each of these mutations induce a particular alteration of the NMJ and characteristic symptoms (presynaptic or postsynaptic). Therefore, the effective therapeutic approach that exists is based on the knowledge that we have about these molecules.

In the last few years, the correct function of NMJs has been identified as one factor involved in motoneuron diseases, a group of neurodegenerative diseases that causes the selective and progressive death of motoneurons. Unfortunately, their molecular mechanisms are hidden under a multifactorial variety of genetic and environmental factors, excitotoxicity, exposure to neurotoxic substances and an elevated prevalence of sporadic cases (Chang and Wu, 2009). Among motoneuron diseases, our laboratory has focused on amyotrophic lateral sclerosis (ALS) in the recent years (Just-Borràs *et al.*, 2019a, 2019b). Several cellular processes are affected in ALS patients and mouse models, including glutamatergic excitotoxicity and oxidative stress, although the precise pathogenesis of the disease remains unknown. ALS animal models show that, before the clinical phase of the disease, some NMJ already degenerate whereas others undergo compensatory reinnervations like nerve sprouting and synaptic remodeling (Dadon-Nachum *et al.*, 2011). On the other hand, we found that the muscles of ALS mice also display an altered molecular pattern at the presymptomatic stage before the onset of the disease (Just-Borràs *et al.*, 2019a), including molecules which have been studied in this thesis, supporting a muscarinic involvement. Thus, in addition to the potential early diagnosis, this data suggests a "die-back" mechanism, which would explain the preceding loss of motor units and muscle weakness before the motoneuron death in the CNS. The muscarinic receptors regulate the repair phenotype of perisynaptic Schwann cells and are overactivated in disease-resistant NMJs (soleus muscle) in $SOD1^{G37R}$ mice (Martineau *et al.*, 2020). Thus, they probably participate in the compensatory mechanisms that protect NMJs early in the onset of ALS disease.

Altogether, this thesis provides *in vivo* data of muscarinic modulation in the skeletal muscle. Identifying how inhibitors of mAChRs, PKA and PKC isoforms work will be important for prospective therapies. For instance, drugs in development depend upon the balance between the various isoenzymes present (Ersvaer *et al.*, 2010; Mochly-Rosen *et al.*, 2012). PKA and PKC isoforms are affected in symptomatic and presymptomatic stages of amyotrophic lateral sclerosis (Just-Borràs *et al.*, 2019a; Lanuza *et al.*, 2019). The dual M_1/M_2 muscarinic modulation could be used to readjust and preserve

the neuromuscular function and muscle strength, being useful for muscular paralysis, fall prevention, aging and neuromuscular disorders such as amyotrophic lateral sclerosis and Duchenne muscular dystrophy. To achieve it, innovative strategies will be needed to create more selective agonists (Bock *et al.*, 2012) and to deliver them as specifically as possible, with techniques like muscarinic photoswitchable modulators (Riefolo *et al.*, 2019).

In this regard, our laboratory is currently working in the new I+D+i project "Effect of neuromuscular activity on the interaction between the muscarinic and neurotrophic synaptic signaling. Role in Amyotrophic Lateral Sclerosis (MAChRALS)" funded by the Spanish *Ministerio de Ciencia, Innovación y Universidades* (PID2019-106332G-B-I00). This will promisingly shed light on how the destabilizers and protectors of NMJ integrity are deregulated in ALS, further investigating the important role of mAChRs. On the other hand, this project will also try to determine whether physical exercise or pharmacological therapy, together with others, can contribute to maintain the appropriate structure and function of the NMJ. Eventually, this good understanding of the molecular pathways in the healthy and ALS-affected skeletal muscle might be useful to potentially correct them and delay the motoneuron failure of the disease. Thus, muscarinic receptors are important modulators of a signaling that is key to maintain the integrity of synapses. Selective agonists and antagonists are promising strategies to treat more specifically and effectively the affected tissues.

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CHAPTER 6

CONCLUSION

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CHAPTER 6

CONCLUSION (English)

Conclusions from Publication 1

Objective-derived conclusions

Objective 1. To determine whether there is interplay between M_1 and M_2 muscarinic acetylcholine receptors (mAChRs) subtypes at the skeletal muscle.

- **M_2 signaling downregulates M_1 protein levels at basal conditions. However, M_1 receptors do not affect M_2 protein levels. This shows that M_2 may act as a brake to balance muscarinic function at the NMJ.**

Objective 2. To determine the specificity of antibodies against PKA catalytic subunits (Cα, Cβ) and regulatory subunits (RIα, RIβ, RIIα, RIIβ).

- **Antibody specificity was validated through knockout cell lysates. The reactivity of the anti-Cα, -Cβ, -RIα, -RIIα antibodies was abolished, respectively, in their specific knockout. We found no cross-reactivity between C subunits or between Rα subunits.**
- **The reactivity of the anti-RIβ, -RIIβ antibodies was absent in 293T and HeLa cell lysates, consistent with reports of minimal to no-expression in those cell lines. Anti-R antibodies showed an unspecific 30 kDa band, which was not affected by any KO.**

Objective 3. To determine whether PKA catalytic (Cα, Cβ) and regulatory (RIβ, RIβ, RIIα, RIIβ) subunits are modulated by M_1 and M_2 mAChRs in the skeletal muscle. Moreover, to determine whether they are regulated by synaptic activity-induced stimulation.

- **M_1 upregulates the PKA RIIβ protein levels.**
- **M_2 downregulates the PKA Cβ and upregulates the RIIα and RIIβ. The M_2 action over Cβ and RIIα needs an active M_1.**
- **Synaptic stimulation increases Cβ protein levels and, under this condition, M_2 still downregulates Cβ.**

Objective 4. To determine whether M_1 and M_2 mAChRs modulate the subcellular translocation of PKA subunits in the skeletal muscle.

- **M_1 receptor constitutively maintains PKA RIα, RIβ, and RIIα in the membrane fraction, whereas the tonic effect of M_2 mAChR, in opposition to M_1, enhances the release of PKA RIβ and RIIα to the cytosol.**

Objective 5. To determine whether M_1 and M_2 mAChRs modulate the protein levels and the subcellular translocation of the scaffold AKAP150.

- **M_1 signaling upregulates AKAP150 protein levels at the NMJ whereas M_2 signaling downregulates them. These regulations take place at the membrane fraction and likely influence PKA subcellular scaffolding.**

Objective 6. To determine PKA subunits that are enriched in the synaptic region of the diaphragm. Moreover, to analyze whether M_2 mAChR affects the interaction between the RIβ subunit and the Cα and Cβ subunits.

- **PKA have a wide cellular distribution at the diaphragm muscle. PKA Cβ and RIβ subunits–but not Cα–are enriched at the synaptic region of the diaphragm.**
- **The action of the M_2 receptor at the diaphragm increases Cβ in both synaptic and extrasynaptic regions.**
- **At the nerve terminal of the NMJ, the tonic action of the M_2 receptor stabilizes the holoenzymes formed by Cα and Cβ with RIβ.**

Objective 7. To determine whether M_1 and M_2 mAChRs modulate the phosphorylation of CREB and SNAP-25 and the involvement of PKA.

- **M_2 action reduces the PKA phosphorylation of the master regulator of gene expression CREB (Ser^{133}) and the essential exocytotic protein SNAP-25 (Thr^{138}) coincident with a decrease in the neurotransmission.**
- **PKA enhances CREB (Ser^{133}) y SNAP-25 (Thr^{138}) phosphorylations at the NMJ. PKA activity is necessary for M_2 to modulate these substrates.**

Conclusion from Publication 2

Objective-derived conclusions

Objective 1. To determine whether the M_1 and M_2 muscarinic acetylcholine receptors (mAChRs) modulate the protein levels and phosphorylation of the presynaptic PKCβI and PKCε isoforms in the skeletal muscle. Moreover, to determine the effect over the widely expressed PKCα.

- **M_1 promotes the priming of both PKCβI and PKCε and downregulates PKCβI protein levels, the latter due to enhanced activity-dependent degradation.**
- **M_2 signaling affects PKCε through two different pathways: a PKA-dependent pathway where M_2 downregulates PKCε; and a PKA-independent pathway where M_2 induces PKCε maturation through PDK1 (See Objective 3). Conversely, M_2 does not modulate PKCβI.**
- **M_2 mAChR–but not M_1–downregulates PKCα levels and priming, which could be associated with a reduction of the postsynaptic responsiveness.**

Objective 2. To determine whether M_1 and M_2 mAChRs modulate the subcellular translocation of PKCβI and PKCε and their phosphorylated forms at the NMJ.

- **M_1-induced PKCβI priming and downregulation occur in the membrane fraction. M_1 translocates PKCε to the cytosol fraction.**
- **M_2-induced PKCε priming and downregulation occur in the membrane fraction. M_2 does not affect the PKCβI translocation.**

Objective 3. To determine whether PDK1 phosphorylation is induced by M_1 and M_2 mAChRs and whether PDK1 activity is linked to PKCβI and PKCε maturation and the phosphorylation of their substrates at the NMJ.

- **Both M_1 and M_2 induce PDK1 (Ser^{241}) phosphorylation.**
- **PDK1 activity promotes the priming of both PKCβI and PKCε and downregulates PKCβI protein levels, the latter due to enhanced activity-dependent degradation.**
- **PDK1 action is linked to either M_1 or M_2 receptors, promoting the PKC-phosphorylation of SNAP-25 (Ser^{187}) and MARCKS ($Ser^{152/156}$) and downregulating Munc18-1 (Ser^{313}) phosphorylation.**

Objective 4. To determine whether M_1 and M_2 mAChRs modulate the protein levels and phosphorylation of the PKC substrates Munc18-1, SNAP-25 and MARCKS.

- **M_1 enhances the PKC-phosphorylation of Munc18-1, SNAP-25 and MARCKS without affecting their protein levels.**
- **M_2 downregulates Munc18-1 phosphorylation and protein levels, MARCKS phosphorylation and does not affect SNAP-25 PKC-phosphorylation (Ser^{187}).**

Objective 5. To determine the role of PKCβI, PKCε and PKA in each modulation of Munc18-1, SNAP-25 and MARCKS by M_1 and M_2 muscarinic receptors subtypes.

- **The activity of both PKCβI and PKCε is a requirement for M_1 mAChR to induce the phosphorylation of Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}) and MARCKS ($Ser^{152/156}$).**
- **The PKA activity does not participate in the M_1 enhancement of Munc18-1 and MARCKS phosphorylation, but it is required to maintain MARCKS levels and for the PKC-phosphorylation of SNAP-25 (Ser^{187}).**

- **The activity of PKCε necessary for M_2 to inhibit MARCKS ($Ser^{152/156}$) phosphorylation and Munc18-1 (Ser^{313}) phosphorylation and expression. Conversely, PKCβI activity is not required for M_2.**
- **The PKA activity is a requirement for M_2 to downregulate Munc18-1 phosphorylation and expression. When PKA is blocked, M_2 signaling shifts to promote Munc18-1 and MARCKS phosphorylation, visualizing the effect of its PKA-independent pathway.**

Objective 6. To determine whether M_1 and M_2 mAChRs modulate the translocation of Munc18-1, SNAP-25 and MARCKS at the NMJ.

- **The M_1 signaling enhances Munc18-1, MARCKS and SNAP-25 phosphorylations at the membrane fraction. M_1 also induces the translocation of Munc18-1 from the cytosol to the membrane fraction.**
- **The M_2 signaling inhibits MARCKS phosphorylation and Munc18-1 phosphorylation and expression at the membrane fraction.**

Objective 7. To determine whether the signaling of M_2 mAChR over PKCβI, PKCε, Munc18-1 and SNAP-25 protein level and phosphorylation occurs at the synaptic region of the rat diaphragm.

- **PKCβI, PKCε, Munc18-1 and SNAP-25 are highly enriched at the synaptic region of the diaphragm, in consistency with their presynaptic location. Munc18-1 was more abundant than the others in the extrasynaptic region, in concordance with its presence in motor axons, which extend outside the synaptic region.**
- **The M_2–PKCε–Munc18-1 pathway only occurs at the synaptic region of the diaphragm. Conversely M_2 does not affect PKCβI or SNAP-25 in any region.**

Objective 8. To corroborate the location of NMJ nerve terminal of the PKCε, PKCβI, PDK1, Munc18-1 and SNAP-25.

- **PKCβI and PKCε staining is limited to the presynaptic region over nAChRs and does not surpass areas outside the NMJ plate, in concordance with previous studies.**
- **PDK1 staining appears finely punctuated over nAChRs without surpassing areas outside the NMJ plate, in concordance with previous studies.**
- **Munc18-1 is expressed at the presynaptic component of the NMJ and in axon terminals. SNAP-25 is exclusively located in the presynaptic component of the NMJ. Optical sections corroborate that Munc18-1 and SNAP-25 are concentrated presynaptically over the postsynaptic gutters, in concordance with previous studies.**

Main conclusion

This thesis provides molecular data evidence to the muscarinic signaling that achieves the modulation of the ACh release at the NMJ, which is that M_1 increases and M_2 decreases ACh release.

At the NMJ, M_1 enhances PKC maturation and activity. First, M_1 induces PDK1 phosphorylation, whose activity promotes PKCβI and PKCε maturation(■ Figure 20). On the other hand, the M_1/G_q/PLCβ and calcium mobilization pathway activates PKCβI and its subsequent degradation. M_1 involves both PKCβI and PKCε isoforms to phosphorylate Munc18-1 (Ser^{313}), MARCKS ($Ser^{152/156}$) and SNAP-25 (Ser^{187}), the latter also requiring PKA. The three phosphorylated substrates are recruited to the membrane, where they likely participate in neurotransmission.

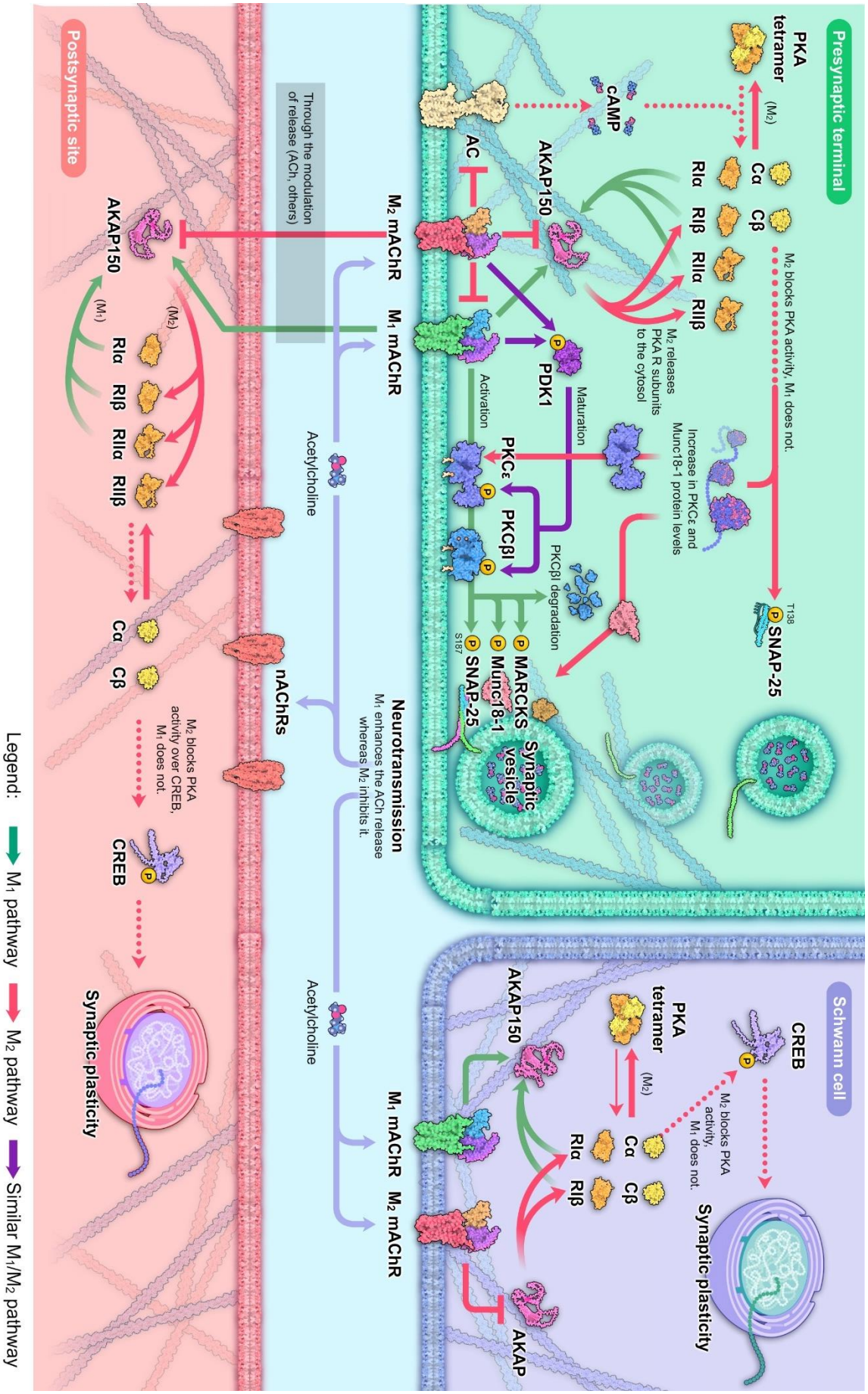
On the other hand, the M_2 inhibits PKA, ubiquitously expressed in the three cell components of the NMJ. M_2 inhibits Cβ protein levels and increases RIIα and RIIβ. Secondly, M_2 modulates PKA translocation, possibly related to an AKAP150 downregulation, which liberates RIβ and RIIα to the cytosol, where C subunits are abundant. This M_2 signaling promotes the interaction between RIβ:Cα and RIβ:Cβ at the NMJ. Lastly, M_2 decreases SNAP-25 Thr^{138} phosphorylation (PKA), in nerve terminals, and CREB (Ser^{133}), in postsynaptic myocytes and Schwann cells. These effects might be related to its well-known downregulation of neurotransmission.

We determined several interactions between M_1 and M_2 pathways. M_1 affects M_2/PKA by increasing AKAP150 and recruiting RIα, RIβ and RIIα to

the membrane. This appears to counter-balance the actions of M_2, although M_1 *per se* does not affect any PKA-substrate studied. M_2 over M_1/PKC downregulates M_1 and uses two different pathways over PKC: a PKA-dependent pathway where M_2 inhibits PKCε and Munc18-1 protein levels; and a PKA-independent pathway, similar to M_1, where M_2 promotes PDK1 phosphorylation and PKCε maturation at the membrane compartment. M_2 signaling *per se* inhibits the phosphorylation of Munc18-1 and MARCKS. Blocking PKA leaves only its PKA-independent pathway, which shifts M_2 signaling to enhance Munc18-1 and MARCKS phosphorylation like M_1.

Therefore, the PKA and PKC pathways are complementarily balanced by M_1 and M_2 mAChRs signalings to maintain and adjust the synaptic vesicle release machinery to the neurotransmission at the NMJ.

Figure 20. **Concluding muscarinic signaling at the NMJ studied in this thesis.** The muscarinic receptors (mAChRs) M_1 and M_2 are located in the presynaptic nerve terminal (green cell, left) and the Schwann cells (blue cell, right), with a single report of postsynaptic M_1 mAChRs. The studied PKC pathway is specific of presynaptic terminals, thanks to the exclusive expression of the PKCβI, PKCε and their SNARE/SM substrates. Contrarily, the PKA pathway extends to the three cell components of the NMJ due to the ubiquity of PKA subunits (Cα, Cβ, RIα, RIβ, RIIα, RIIβ). All R subunits have been represented in the three cell components due to the ubiquity and to represent clearly their participation.
M_1 signaling (►green arrows) uses Gq/PLCβ/calcium to activate the PKCβI and PKCε, which causes the activity-dependent degradation of PKCβI. Both PKC isoforms participate in the phosphorylation of MARCKS, Munc18-1 and SNAP-25 Ser^{187} (PKC) at the membrane, which likely contribute to enhance the neurotransmission.
The M_2 signaling (►red arrows) inhibits adenylate cyclase (AC) and cAMP levels (pathways decreased by M_2 are dotted). We found three mechanisms where M_2 likely reduces PKA activity. First, M_2 inhibits the protein level of Cβ and increases RIIα and RIIβ. Secondly, M_2 decreases AKAP150 levels and releases RIβ and RIIα to the cytosol, where C subunits are abundant. Thirdly, M_2 enhances the R:C interaction between RIβ, Cα and Cβ at the NMJ. All these explain how M_2 inhibits the phosphorylation of SNAP-25 Thr^{138} (PKA), in nerve terminals, and CREB (Ser^{133}), in postsynaptic myocytes and Schwann cells. These changes might be related to a decrease in neurotransmission.
Finally, the M_1 and M_2 pathways crosstalk at some points. M_1 over PKA increases AKAP150 and recruits RIα, RIβ and RIIα to the membrane. Regarding M_2 over PKC, M_2 decreases the levels of M_1, PKCε and Munc18-1, the latter two involving PKA activity (M_2 does not affect PKCβI or SNAP-25 Ser^{187}). This decreases in Munc18-1 and MARCKS phosphorylation at the membrane fraction and likely contributes to the decrease in neurotransmission. Finally, both M_1 and M_2 cooperatively activate PDK1 (►purple arrows). PDK1 itself increases PKCβI and PKCε maturation and produces effects at the crossroads between M_1 (over SNAP-25 and MARCKS) and M_2 (over Munc18-1). For M_1, it is a second path to enhance and maintain the PKC pool. For M_2, it is a PKA-independent pathway still observable after PKA is blocked. When all PKA is blocked, M_2 shifts to promote PKCε, Munc18-1 and MARCKS phosphorylation, likely contributing to enhance neurotransmission. *Source: own elaboration.*


Presynaptic terminal
PKA tetramer
(M2)
Cα
Cβ
RIα
RIβ
RIIα
RIIβ
cAMP
AC
AKAP150
M2 blocks PKA activity, M1 does not.
M2 releases PKA R subunits to the cytosol
Increase in PKCε and Munc18-1 protein levels
PDK1
Maturation
Activation
PKCε
PKCβI
PKCβI degradation
T138
SNAP-25
S187
MARCKS
Munc18-1
SNAP-25
Synaptic vesicle
Neurotransmission
M1 enhances the ACh release whereas M2 inhibits it.
Acetylcholine
M2 mAChR
M1 mAChR
Through the modulation of release (ACh, others)
nAChRs
Postsynaptic site
AKAP150
(M1)
(M2)
RIα
RIβ
RIIα
RIIβ
Cα
Cβ
M2 blocks PKA activity over CREB. M1 does not.
CREB
Synaptic plasticity
Schwann cell
CREB
PKA tetramer
(M2)
M2 blocks PKA activity, M1 does not.
Synaptic plasticity
AKAP150
AKAP
Legend:
M1 pathway
M2 pathway
Similar M1/M2 pathway

CONCLUSIÓ (Català)

Conclusions de la Publicació 1

Conclusions segons els objectius

Objectiu 1. Determinar si existeix cap interacció entre els subtipus de receptors muscarínics d'acetilcolina M_1 i M_2 en el múscul esquelètic.

- **La senyalització M_2 disminueix els nivells de proteïna de M_1 en condicions basals. En canvi, el receptor M_1 no afecta els nivells de M_2. Això indica que el M_2 podria actuar com a fre per equilibrar la funció muscarínica en la unió neuromuscular (UNM).**

Objectiu 2. Determinar l'especificitat dels anticossos vers les subunitats catalítiques de PKA (Cα, Cβ) i les subunitats reguladores (RIα, RIβ, RIIα, RIIβ).

- **L'especificitat dels anticossos ha estat validada mitjançant lisats de cèl·lules genoanul·lades (*knockout*). La reactivitat dels anticossos anti-Cα, -Cβ, -RIα, -RIIα només és abolida en el seu *knockout* específic. No hem observat reactivitat creuada entre subunitats C ni entre subunitats Rα.**
- **Els anticossos anti-RIβ i anti-RIIβ no mostren reactivitat en els lisats cel·lulars 293T ni HeLa, en concordança amb estudis que senyalen expressió mínima o nul·la en aquestes línies cel·lulars. Hem observat una banda inespecífica de 30 kDa en els anticossos anti-R que no és afectada per cap KO.**

Objectiu 3. Determinar si les subunitats catalítiques (Cα, Cβ) i reguladores (RIβ, RIβ, RIIα, RIIβ) estan modulades pels receptors M_1 i M_2 en el múscul esquelètic. A més, determinar si aquestes subunitats estan regulades per l'activitat sinàptica induïda mitjançant estimulació.

- **M_1 incrementa els nivells de proteïna de PKA RIIβ.**
- **M_2 disminueix PKA Cβ i incrementa RIIα i RIIβ. L'acció de M_2 sobre Cβ i RIIα necessita que M_1 estigui actiu.**
- **L'activitat sinàptica augmenta els nivells de proteïna de Cβ i, en aquesta condició, M_2 segueix disminuint els nivells de Cβ.**

Objectiu 4. Determinar si els receptors M_1 i M_2 modulen la translocació subcel·lular de les subunitats de PKA en el múscul esquelètic.

- **El receptor M_1 manté constitutivament PKA RIα, RIβ, i RIIα en la fracció de membrana, mentre que l'efecte tònic del receptor M_2 allibera la PKA RIβ i la RIIα cap al citosol.**

Objectiu 5. Determinar si els receptors M_1 i M_2 modulen els nivells de proteïna i la translocació subcel·lular de la proteïna de bastida AKAP150.

- **La senyalització del receptor M_1 incrementa els nivells de proteïna de l'AKAP150 a la UNM, mentre que la senyalització M_2 els disminueix. Aquests canvis ocorren en la fracció de membrana i concorden amb la localització subcel·lular de les subunitats de PKA.**

Objectiu 6. Determinar quines subunitats de PKA són més abundants en la regió sinàptica del diafragma. Analitzar si el receptor M_2 afecta a la interacció entre la subunitat RIβ i les subunitats Cα i Cβ.

- **La PKA té una amplia distribució cel·lular en el múscul diafragma. Les subunitats de PKA Cβ i RIβ –però no Cα– estan enriquides a la regió sinàptica del diafragma.**
- **L'activitat del receptor M_2 augmenta els nivells de Cβ tant a la regió sinàptica como l'extrasinàptica del diafragma.**
- **En el terminal nerviós de la UNM, l'actividad del receptor M_2 estabilitza els holoenzims formats per Cα i Cβ amb RIβ.**

Objectiu 7. Determinar si M_1 i M_2 mAChRs modulen la fosforilació de CREB i SNAP-25 i la implicació de PKA.

- **L'acció de M_2 disminueix la fosforilació de PKA del regulador de la expressió gènica CREB (Ser^{133}) i de la proteïna exocitòtica SNAP-25 (Thr^{138}), coincidint amb una disminució en la neurotransmissió.**
- **La PKA promou la fosforilació dels substrats CREB (Ser^{133}) i SNAP-25 (Thr^{138}) a la UNM. L'activitat de la PKA és necessària perquè M_2 pugui modular ambdós substrats.**

Conclusions de la Publicació 2

Conclusions segons els objectius

Objectiu 1. Determinar si els receptors muscarínics d'acetilcolina M_1 i M_2 modulen els nivells de proteïnes i la fosforilació de les isoformes presinàptiques PKCβI i PKCε en el múscul esquelètic. A més, determinar el seu efecte sobre la isoforma PKCα, expressada de fomra ubiqua.

- **M_1 promou la maduració de PKCβI i PKCε i disminueix els nivells de proteïna PKCβI, aquesta última disminució a causa de la seva degradació dependent d'activitat.**
- **M_2 afecta la PKCε a través de dues vies diferents: una via dependent de PKA, per la que M_2 disminueix la PKCε; i una via independent de PKA, per la que M_2 indueix la maduració de PKCε a través de PDK1 (Veure Objectiu 3). Por el contrario, M_2 no modula PKCβI.**
- **El receptor M_2 –però no el M_1– afecta la PKCα disminuint els seus nivells i fosforilació, el que podria estar relacionat amb una reducció de la resposta postsinàptica.**

Objectiu 2. Determinar si els receptors M_1 i M_2 modulen la translocació subcel·lular de la PKCβI, la PKCε i les seves formes fosforilades en la UNM.

- **El receptor M_1 indueix la maduració i la disminució dels nivells de PKCβI a la fracció de membrana. M_1 promou la translocació de PKCε cap al citosol.**
- **El receptor M_2 indueix la maduració i la disminució dels nivells de PKCε a la fracció de membrana. M_2 no afecta la translocació de PKCβI.**

Objectiu 3. Determinar si la fosforilació de PDK1 és induïda pels receptors M_1 i M_2 i si l'activitat de la PDK1 està associada a la maduració de PKCβI i PKCε i a la fosforilació dels seus substrats a la UNM.

- **Tant el M_1 com el M_2 indueixen la fosforilació de la PDK1 (Ser^{241}).**
- **L'activitat de la PDK1 promou la maduració de la PKCβI i la PKCε i disminueix els nivells de PKCβI, aquesta última disminució a causa de la degradació dependent d'activitat.**
- **L'acció de PDK1 per sí mateixa promou la fosforilació PKC dels substrats SNAP-25 (Ser^{187}) i MARCKS ($Ser^{152/156}$) i disminueix la fosforilació de Munc18-1 (Ser^{313}).**

Objectiu 4. Determinar si els receptors M_1 i M_2 modulen els nivells de proteïna i fosforilació dels substrats de PKC Munc18-1, SNAP-25 i MARCKS.

- **M_1 afavoreix la fosforilació de PKC dels tres substrats Munc18-1, SNAP-25 i MARCKS sense afectar els seus nivells de proteïna.**
- **M_2 inhibeix la fosforilació de Munc18-1 i els seus nivells de proteïna, inhibeix la fosforilació de MARCKS i no afecta la fosforilació PKC de SNAP-25 (Ser^{187}).**

Objectiu 5. Determinar el paper de PKCβI, PKCε i PKA en cada modulació dels receptors M_1 i M_2 sobre Munc18-1, SNAP-25 i MARCKS.

- **M_1 necessita ambdues PKCβI i PKCε per promoure la fosforilació de Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}) i MARCKS ($Ser^{152/156}$).**
- **M_1 no necessita la PKA per promoure la fosforilació de Munc18-1 ni de MARCKS, però sí que la requereix per mantenir els nivells de MARCKS i per la fosforilació PKC de SNAP-25 (Ser^{187}).**

- **M_2 necessita la PKCε per poder inhibir la fosforilació de MARCKS ($Ser^{152/156}$) i la fosforilació i expressió de Munc18-1 (Ser^{313}). D'acord amb els resultats anteriors, M_2 no necessita la PKCβI.**
- **M_2 requereix la PKA per disminuir la fosforilació i expressió de Munc18-1. Quan la PKA està bloquejada, la senyalització del receptor M_2 canvia per augmentar la fosforilació de Munc18-1 i MARCKS, manifestant l'efecte de la seva via independent de PKA.**

Objectiu 6. Determinar si els receptores M_1 i M_2 modulen la translocació de Munc18-1, SNAP-25 i MARCKS a la UNM.

- **La senyalització de M_1 afavoreix la fosforilació de Munc18-1, MARCKS i SNAP-25 en la fracció de membrana. M_1 també indueix la translocació de Munc18-1 des del citosol cap a la membrana.**
- **La senyalització de M_2 inhibeix la fosforilació de MARCKS i la fosforilació i expressió de Munc18-1 en la fracció de membrana.**

Objectiu 7. Determinar si la senyalització de M_2 mAChR sobre els nivells i la fosforilació de PKCβI, PKCε, Munc18-1 i SNAP-25 ocorre a la regió sinàptica del diafragma.

- **PKCβI, PKCε, Munc18-1 i SNAP-25 estan altament enriquits a la regió sinàptica del diafragma, en concordància amb la seva localització presinàptica. D'aquestes proteïnes, Munc18-1 és la que mostra més reactivitat a la regió extrasinàptica, d'acord amb la seva presència en els axons motors, que s'estenen fora de la regió sinàptica.**
- **La via M_2–PKCε–Munc18-1 només es detecta en la regió sinàptica del diafragma. M_2 no afecta a la PKCβI ni el SNAP-25 en cap regió.**

Objectiu 8. Corroborar la localització de las proteïnes PKCε, PKCβI, PDK1, Munc18-1 i SNAP-25 en el terminal nerviós de la UNM.

- **El marcatge de PKCβI i PKCε es limita a la regió presinàptica, per sobre dels receptors nicotínics i no excedeix àrees fora de la placa de la UNM, en concordança amb els estudis previs.**
- **El marcatge de PDK1 apareix finament puntuat sobre dels receptors nicotínics sense sobrepassar àrees fora de la placa de la UNM, en concordança amb estudis previs.**
- **Munc18-1 s'expressa en el terminal i l'axó preterminal de la UNM. SNAP-25 es troba exclusivament en el component presinàptic de la UNM. Mitjançant seccions òptiques, hem corroborat que Munc18-1 i SNAP-25 se concentren presinàpticament sobre dels plecs postsinàptics, en concordança amb estudis previs.**

Conclusió

Aquesta tesi proporciona dades moleculars de la cascada de senyalització muscarínica que controla la neurotransmissió a la UNM, on M_1 augmenta i M_2 disminueix l'alliberació d'acetilcolina.

M_1 facilita la maduració i l'activitat de les PKC a la UNM. Primer, M_1 indueix la fosforilació de PDK1, l'activitat de la qual promou la maduració de PKCβI i PKCε (■ Figura 20). Per altra banda, la via M_1/G_q/PLCβ i la mobilització de calci desencadenen l'activitat de PKCβI i la seva posterior degradació. M_1 recluta a ambdues isoformes PKCβI i PKCε per afavorir la fosforilació de Munc18-1 (Ser^{313}), MARCKS ($Ser^{152/156}$) i SNAP-25 (Ser^{187}), sent aquesta última la única que requereix activitat PKA. Finalment, els tres substrats fosforilats són reclutats a la membrana, on probablement afavoreixen la neurotransmissió.

D'altra banda, la via M_2 inhibeix la PKA, expressada de forma ubiqua als tres components de la UNM. El M_2 inhibeix els nivells de Cβ i incrementa els de RIIα i RIIβ. Després, M_2 modula la translocació de la PKA, possiblement disminuint AKAP150, el que allibera RIβ i RIIα al citosol, on hi

ha les subunitats C. Aquesta senyalització M_2 promou la interacció entre RIβ:Cα i RIβ:Cβ a la UNM. Finalment, el M_2 disminueix la fosforilació de SNAP-25 Thr^{138} (PKA), als terminals nerviosos, i de CREB (Ser^{133}) al compartiment postsinàptic i les cèl·lules de Schwann. Aquestes regulacions semblen estar d'acord amb la disminució de la neurotransmissió del M_2.

Finalment, hem determinat diverses interaccions entre les vies M_1 i M_2. El M_1 afecta la via M_2/PKA mitjançant l'increment de AKAP150 i la translocació de RIα, RIβ i RIIα a la membrana. Això equilibra les accions del M_2, tot i què M_1 *per se* no afecta cap substrat PKA estudiat. El M_2 respecte la via M_1/PKC inhibeix els nivells de M_1 i utilitza dues vies per modular PKC: la via PKA-dependent inhibeix els nivells de PKCε i Munc18-1; i la via PKA-independent, anàloga a la del M_1, on M_2 indueix la fosforilació de PDK1 i PKCε en la fracció membrana. La via M_2 *per se* inhibeix la fosforilació de Munc18-1 i MARCKS. Bloquejar la PKA revela la via PKA-independent, on M_2 canvia a afavorir la fosforilació de Munc18-1 i MARCKS, de forma similar a la senyalització del M_1.

Per tant, les vies de la PKA i la PKC es troben en equilibri gràcies a les activitats complementàries dels receptors M_1 i M_2, que mantenen i ajusten l'activitat sobre les molècules de la maquinària d'alliberació de vesícules a la UNM.

CONCLUSIÓN (Castellano)

Conclusiones de la Publicación 1

Conclusiones según los objetivos

Objetivo 1. Determinar si existe interacción entre los subtipos de receptores muscarínicos de acetilcolina M_1 y M_2 en el músculo esquelético.

- **La señalización M_2 disminuye los niveles de proteína de M_1 en condiciones basales. Sin embargo, los receptores M_1 no afectan los niveles de M_2. Esto indica que M_2 podría actuar como un freno para equilibrar la función muscarínica en la unión neuromuscular (UNM).**

Objetivo 2. Determinar la especificidad de los anticuerpos frente a las subunidades catalíticas de PKA (Cα, Cβ) y las subunidades reguladoras (RIα, RIβ, RIIα, RIIβ).

- **La especificidad de los anticuerpos ha sido validada a través de lisados de células con genes inactivados (*knockout*). La reactividad de los anticuerpos anti-Cα, -Cβ, -RIα, -RIIα fue abolida, respectivamente, en su *knockout* específico. No encontramos reactividad cruzada entre las subunidades C ni entre subunidades Rα.**
- **Los anticuerpos anti-RIβ y anti-RIIβ no mostraron reactividad en los lisados de células 293T y HeLa, de acuerdo con la expresión mínima o nula en dichas líneas celulares según la literatura. Observamos una banda inespecífica de 30 kDa en los anticuerpos anti-R que no era afectada por ningún KO.**

Objetivo 3. Determinar si las subunidades catalíticas (Cα, Cβ) y reguladoras (RIβ, RIβ, RIIα, RIIβ) están moduladas por los receptores M_1 y M_2 en el músculo esquelético. Además, determinar si dichas subunidades están reguladas por la actividad sináptica inducida mediante estimulación.

- **M_1 incrementa los niveles de proteína PKA RIIβ**
- **M_2 disminuye PKA Cβ e incrementa RIIα y RIIβ. La acción de M_2 sobre Cβ y RIIα necesita que M_1 esté activo.**
- **La actividad sináptica aumenta los niveles de proteína Cβ y, bajo esta condición, M_2 sigue disminuyendo los niveles de Cβ.**

Objetivo 4. Determinar si los receptores M_1 y M_2 modulan la translocación subcelular de las subunidades de PKA en el músculo esquelético.

- **El receptor M_1 mantiene constitutivamente PKA RIα, RIβ, y RIIα en la fracción de membrana, mientras que el efecto tónico del receptor M_2 libera PKA RIβ y RIIα al citosol.**

Objetivo 5. Determinar si los receptores M_1 y M_2 modulan los niveles de proteína y la translocación subcelular de la proteína de anclaje AKAP150.

- **La señalización del receptor M_1 incrementa los niveles de proteína de AKAP150 en la UNM, mientras que la señalización M_2 los disminuye. Estos cambios ocurren en la fracción de membrana y concuerdan con la localización subcelular de PKA.**

Objetivo 6. Determinar las subunidades de PKA más abundantes en la región sináptica del diafragma. Analizar si el receptor M_2 afecta a la interacción entre la subunidad RIβ y las subunidades Cα y Cβ.

- **La PKA tiene una amplia distribución celular en el músculo diafragma. Las subunidades PKA Cβ y RIβ, pero no Cα, están enriquecidas en la región sináptica del diafragma.**
- **La actividad del receptor M_2 aumenta los niveles de Cβ tanto en la región sináptica como en la extrasináptica del diafragma.**
- **En el terminal nervioso de la UNM, la actividad del receptor M_2 estabiliza las holoenzimas formadas por Cα:RIβ y Cβ:RIβ.**

Objetivo 7. Determinar si M_1 y M_2 mAChRs modulan la fosforilación de CREB y SNAP-25 y la implicación de PKA.

- **La acción de M_2 disminuye la fosforilación de PKA del regulador de la expresión génica CREB (Ser^{133}) y de la proteína de exocitosis SNAP-25 (Thr^{138}), coincidiendo con una disminución en la neurotransmisión.**
- **La PKA promueve la fosforilación de los sustratos CREB (Ser^{133}) y SNAP-25 (Thr^{138}) en la UNM. La actividad de PKA es necesaria para que M_2 pueda modular dichos sustratos.**

Conclusiones de la Publicación 2

Conclusiones según los objetivos

Objetivo 1. Determinar si los receptores muscarínicos de acetilcolina M_1 y M_2 modulan los niveles de proteínas y la fosforilación de las isoformas presinápticas PKCβI y PKCε en el músculo esquelético. Además, determinar su efecto sobre la isoforma PKCα, de expresión ubicua.

- **M_1 promueve la maduración de PKCβI y PKCε y disminuye los niveles de proteína PKCβI, esta disminución debido a la degradación dependiente de actividad.**
- **M_2 afecta la PKCε a través de dos vías diferentes: una vía dependiente de PKA, por la que M_2 disminuye la PKCε; y una vía independiente de PKA, por la que M_2 induce la maduración de PKCε a través de PDK1 (Ver Objetivo 3). Por el contrario, M_2 no modula PKCβI.**
- **El receptor M_2 –pero no M_1– afecta la PKCα disminuyendo sus niveles y fosforilación, lo que podría estar relacionado con una reducción de la respuesta postsináptica.**

Objetivo 2. Determinar si los receptores M_1 y M_2 modulan la translocación subcelular de PKCβI, PKCε y sus formas fosforiladas en la UNM.

- **El receptor M_1 induce la maduración y disminución de los niveles de PKCβI en la fracción de membrana. M_1 transloca PKCε a la fracción del citosol.**
- **El receptor M_2 induce la maduración y disminución de los niveles de PKCε en la fracción de membrana. M_2 no afecta la translocación de PKCβI.**

Objetivo 3. Determinar si la fosforilación de PDK1 es inducida por los receptores M_1 y M_2 y si la actividad de PDK1 está vinculada a la maduración de PKCβI y PKCε y a la fosforilación de sus sustratos en la UNM.

- **Tanto M_1 como M_2 inducen la fosforilación de PDK1 (Ser^{241}).**
- **La actividad de PDK1 promueve la maduración de PKCβI y PKCε y disminuye los niveles PKCβI, esta disminución debido a la degradación dependiente de actividad.**
- **La acción de PDK1 por sí misma promueve la fosforilación PKC de SNAP-25 (Ser^{187}) y MARCKS ($Ser^{152/156}$) y disminuye la fosforilación de Munc18-1 (Ser^{313}).**

Objetivo 4. Determinar si los receptores M_1 y M_2 modulan los niveles de proteína y fosforilación de los sustratos de PKC Munc18-1, SNAP-25 y MARCKS.

- **M_1 favorece la fosforilación de PKC de los tres sustratos Munc18-1, SNAP-25 y MARCKS sin afectar sus niveles de proteína.**
- **M_2 inhibe la fosforilación de Munc18-1 y su nivel de proteína, inhibe la fosforilación de MARCKS y no afecta a la fosforilación PKC de SNAP-25 (Ser^{187}).**

Objetivo 5. Determinar el papel de PKCβI, PKCε y PKA en cada modulación de los receptores M_1 y M_2 sobre Munc18-1, SNAP-25 y MARCKS.

- **M_1 necesita a ambas PKCβI y PKCε para promover la fosforilación de Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}) y MARCKS ($Ser^{152/156}$).**
- **M_1 no necesita PKA para promover la fosforilación de Munc18-1 y MARCKS, pero sí que se requiere para mantener los niveles de MARCKS y para la fosforilación PKC de SNAP-25 (Ser^{187}).**

- **M_2 necesita la PKCε para poder inhibir la fosforilación de MARCKS ($Ser^{152/156}$) y la fosforilación y expresión de Munc18-1 (Ser^{313}). Por el contrario, M_2 no necesita PKCβI.**
- **M_2 requiere PKA para disminuir la fosforilación y expresión de Munc18-1. Cuando la PKA está bloqueada, el receptor M_2 cambia para aumentar la fosforilación de Munc18-1 y MARCKS, manifestando el efecto de su vía independiente de PKA.**

Objetivo 6. Determinar si los receptores M_1 y M_2 modulan la translocación de Munc18-1, SNAP-25 y MARCKS en la UNM.

- **La señalización de M_1 favorece la fosforilación de Munc18-1, MARCKS y SNAP-25 en la fracción de membrana. M_1 también induce la translocación de Munc18-1 del citosol a la membrana.**
- **La señalización de M_2 inhibe la fosforilación de MARCKS y la fosforilación y expresión de Munc18-1 en la fracción de membrana.**

Objetivo 7. Determinar si la señalización de M_2 mAChR sobre los niveles y la fosforilación de PKCβI, PKCε, Munc18-1 y SNAP-25 ocurre en la región sináptica del diafragma.

- **PKCβI, PKCε, Munc18-1 y SNAP-25 están altamente enriquecidos en la región sináptica del diafragma, en concordancia con su ubicación presináptica. De estas proteínas, Munc18-1 muestra mayor reactividad en la región extrasináptica, de acuerdo con su presencia en los axones motores, que se extienden fuera de la región sináptica.**
- **La vía M_2–PKCε–Munc18-1 sólo se detecta en las muestras de región sináptica del diafragma. Por el contrario, M_2 no afecta a PKCβI o SNAP-25 en ninguna región.**

Objetivo 8. Corroborar la localización en el terminal nervioso de la UNM de las proteínas PKCε, PKCβI, PDK1, Munc18-1 y SNAP-25.

- **El marcaje de PKCβI y PKCε se limita a la región presináptica, sobre los receptores nicotínicos y no excede áreas fuera de la placa de la UNM, en concordancia con estudios previos.**
- **El marcaje de PDK1 aparece finamente puntuado sobre los receptores nicotínicos sin sobrepasar áreas fuera de la placa de la UNM, en concordancia con estudios previos.**
- **Munc18-1 se expresa en el terminal y en el axón preterminal de la UNM. SNAP-25 se encuentra exclusivamente en el componente presináptico de la UNM. Mediante secciones ópticas, hemos corroborado que Munc18-1 y SNAP-25 se concentran presinápticamente sobre los pliegues postsinápticos, en concordancia con estudios previos.**

Conclusión

Esta tesis proporciona datos moleculares de la cascada de señalización muscarínica que controla la neurotransmisión en la UNM, que es que M_1 aumenta y M_2 disminuye la liberación de acetilcolina.

M_1 facilita la maduración y la actividad de las PKC de la UNM. Primero, M_1 induce la fosforilación de PDK1, cuya actividad promueve la maduración de PKCβI y PKCε. Por otro lado, la vía $M_1/G_q/PLC\beta$ y la movilización de calcio desencadenan la actividad de PKCβI y su posterior degradación. M_1 recluta a ambas isoformas PKCβI y PKCε para favorecer la fosforilación de Munc18-1 (Ser^{313}), MARCKS ($Ser^{152/156}$) y SNAP-25 (Ser^{187}), siendo esta última la única que requiere actividad PKA. Finalmente, los tres sustratos fosforilados son reclutados a la membrana, donde probablemente favorecen la neurotransmisión.

Por otro lado, la señalización del receptor M_2 inhibe la PKA, expresada de forma ubicua en los tres componentes de la UNM. M_2 inhibe los niveles de Cβ e incrementa los de RIIα y RIIβ. Además, M_2 modula la translocación de PKA, posiblemente disminuyendo AKAP150, lo que libera las subunidades RIβ y RIIα al citosol con las subunidades C. Esta señalización favorece la interacción entre RIβ:Cα y RIβ:Cβ en la UNM. Finalmente, M_2

disminuye la fosforilación de SNAP-25 Thr^{138} (PKA), en los terminales nerviosos, y de CREB (Ser^{133}) en el compartimento postsináptico y las células de Schwann. Estas regulaciones parecen estar en línea con la conocida disminución de la neurotransmisión de M_2.

Finalmente, hemos determinado varias interacciones entre las vías M_1 y M_2. M_1 afecta la vía M_2/PKA incrementando AKAP150 y reclutando RIα, RIβ y RIIα hacia la membrana. A pesar de esto, M_1 *per se* no afecta ningún sustrato de PKA de los estudiados. El M_2 respeto la vía M_1/PKC inhibe los niveles del propio M_1 y utiliza dos vías para modular PKC: una vía dependiente de PKA inhibe los niveles de PKCε y Munc18-1; y una vía PKA-independente, similar a la de M_1, donde M_2 induce la fosforilación de PDK1 y PKCε en la fracción membrana. M_2 *per se* inhibe la fosforilación de Munc18-1 y MARCKS. Al bloquear la PKA, se observa la vía PKA-independiente, donde M_2 cambia para favorecer la fosforilación de Munc18-1 y MARCKS, de forma similar a la señalización de M_1.

Por lo tanto, las vías de la PKA y la PKC se encuentran en equilibrio gracias a las actividades complementarias de los receptores M_1 y M_2, que mantienen y ajustan su actividad sobre las moléculas de la maquinaria de liberación de vesículas sinápticas en la UNM.

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CHAPTER 7

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Víctor Cilleros Mañé

APPENDIX

SCIENTIFIC CONTRIBUTIONS

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APPENDIX

SCIENTIFIC CONTRIBUTIONS

Publications derived from the present work:

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Authors: Just L, Hurtado E, **Cilleros V**, Biondi O, Charbonnier F, Tomàs M, Garcia N, Lanuza MA, Tomàs JM.
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Authors: Garcia N; Lanuza MA; Nadal N; Tomàs M; **Cilleros-Mañé V**; Just-Borràs L; Tomàs J.
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Title: Physical Exercise Improves the Altered BDNF-NT4/TrkB Signaling In The Neuromuscular Junction Of Amyotrophic Lateral Sclerosis Mice
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Title: Neuromuscular Activity Regulates PKA Catalytic and Regulatory Subunits and its Downstream Signaling Pathway For ACh Release at the NMJ
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Authors: Guiu-Ortin M; Just-Borràs L; **Cilleros-Mañé V**; Polishchuk A; Fenoll-Brunet R.
Title: Promoting student engagement for a global multicultural environment: A Case Study
Format: Poster

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