Polishchuk *et al. Cell Communication and Signaling* (2025) 23:437
https://doi.org/10.1186/s12964-025-02440-4

Cell Communication and Signaling

RESEARCH Open Access

Activity-dependent muscarinic signalling regulates presynaptic PKC pathway and neurotransmission machinery at the neuromuscular junction

Aleksandra Polishchuk[1,2], Laia Just-Borràs[1,2], Víctor Cilleros-Mañé[1,2], Marta Balanyà-Segura[1,2], Carolina Silvera Simón[1,2], Marta Tomàs[1,2], Meryem Jami El Hirchi[1,2], Marina Díaz-Wallach[1,2], Erica Hurtado[1,2†], Josep Tomàs[1,2†] and Maria A. Lanuza[1,2*†]

Abstract

Background Acetylcholine (ACh) signalling mediated by muscarinic acetylcholine receptors (mAChRs) significantly influences various physiological functions, including muscle contraction. Among these receptors, the M_1 and M_2 subtypes are key modulators of neurotransmission at the neuromuscular junction (NMJ), acting through the protein kinase C (PKC) signalling pathway, critical for synaptic function. PKC phosphorylates essential synaptic vesicle-associated proteins such as Mammalian uncoordinated-18 (Munc18-1) and Synaptosome-associated protein of 25 kDa (SNAP-25), facilitating synaptic vesicle fusion and neurotransmitter release. Although neurotransmitter release at the NMJ is known to be coordinately regulated by both presynaptic nerve stimulation and retrograde signalling from muscle contraction, the role of these activities in controlling M_1 and M_2 receptor-mediated PKC pathways has not been previously explored.

Methods To differentiate the effects of presynaptic nerve stimulation from muscle contraction on M_1 and M_2 receptor pathways, rat phrenic nerves were electrically stimulated at 1 Hz for 30 min, both with and without muscle contraction, which was abolished with μ-conotoxin GIIIB. Selective inhibitors Pirenzepine (M_1 receptor antagonist) and Methoctramine (M_2 receptor antagonist) were used to differentiate the muscarinic receptor-specific PKC pathways. Protein levels and phosphorylation states were analysed through Western blotting. Immunohistochemical analysis was used to locate specific molecules at the NMJ.

Results We observed that presynaptic nerve stimulation resulted in a downregulation of M_2 receptor expression, while nerve-induced muscle contraction upregulated both M_1 and M_2 receptors. Interestingly, M_1 receptor activity was consistently inhibited by the M_2 receptor signalling. Regarding PKC signalling, our data revealed that M_1 receptor

†Erica Hurtado, Josep Tomàs and Maria A. Lanuza contributed equally to this work.

*Correspondence:
Maria A. Lanuza
mariaangel.lanuza@urv.cat

Full list of author information is available at the end of the article

activity selectively downregulated PKCβ1, whereas M_2 receptor activity reduced PKCε expression under both presynaptic stimulation and nerve-induced muscle contraction. These changes influenced the phosphorylation of key PKC substrates, Munc18-1 and SNAP-25, essential for synaptic vesicle dynamics. Specifically, the M_1-PKCβ1 axis was critical for Munc18-1 phosphorylation during presynaptic activity, thus regulating synaptic vesicle fusion. Meanwhile, the M_2-PKCε pathway, modulated by M_1 receptor activity, controlled SNAP-25 phosphorylation during both presynaptic activity and nerve-induced muscle contraction, ultimately impacting neurotransmitter release.

Conclusion Our findings reveal a detailed molecular mechanism underlying the activity-dependent interplay between M_1 and M_2 mAChRs and their respective PKC isoforms, highlighting their combined roles in optimizing acetylcholine release at the NMJ.

Introduction

The neuromuscular junction (NMJ) is a synapse that connects motor neurons to muscle fibres, providing the transmission of signals that activate postsynaptic nicotinic acetylcholine receptors, allowing efficient neurotransmission from nerve to muscle, which is essential for muscle contraction and movement. Within this specialized synapse, presynaptic muscarinic acetylcholine receptors (mAChRs) and protein kinase C (PKC) play central roles in modulating neurotransmission and synaptic plasticity [1–9].

mAChRs, members of the G protein-coupled receptor family, are activated by acetylcholine released from motor neurons themselves and mediate diverse effects on NMJ function. Among the five known mAChR subtypes, M_1 ($G\alpha_q$-type) and M_2 ($G\alpha_i$-type) [10] are the most prominently expressed at the NMJ [11, 12]. Activation of M_1 mAChRs enhances acetylcholine release, while M_2 mAChRs typically inhibit it, ensuring precise control over muscle contraction [6, 13]. The functional interplay between these mAChR subtypes at the presynaptic terminal of the NMJ significantly influences synaptic transmission dynamics, contributing to accurate motor control.

PKC, a family of serine/threonine kinases, also serves as a central regulator of synaptic transmission and plasticity at the NMJ [14, 15]. Activation of PKC isoforms is subtypes-dependent: occurs in response to calcium influx, diacylglycerol production, and phosphatidylserine for classical PKC isoforms (cPKCs), whereas novel PKC isoforms (nPKCs) are activated by diacylglycerol and phosphatidylserine but are calcium-dependent [16]. Once activated, PKC phosphorylates a variety of substrates, including proteins essential for neurotransmitter release and synaptic vesicle trafficking. At the NMJ, PKCβ1 and PKCε are the predominant isoforms localized presynaptically, where they play significant roles in regulating the release of acetylcholine (ACh) [2, 4]. Notably, their activity is influenced by the muscarinic receptors [9], linking mAChR signalling to PKC-mediated regulation of neurotransmission.

Munc18-1 (mammalian uncoordinated-18) and SNAP-25 (synaptosome-associated protein 25) are two presynaptic PKC targets, critical for the neurotransmission at the NMJ. Munc18-1, localized in the presynaptic terminal [7, 9], facilitates the assembly of the SNARE complex, which is essential for synaptic vesicle fusion and increases the pool available for release [17, 18]. Specific phosphorylation of Munc18-1 by PKC at residues such as serine 306 (S306) and serine 313 (S313) can significantly modulate its interaction with syntaxin-1 and influence synaptic vesicle exocytosis [19–22]. At NMJ, the PKCβ1 and PKCε isoforms work together to regulate the activity-dependent phosphorylation of Munc18-1 [7], linking presynaptic signalling pathways to fine-tuned modulation of neurotransmitter release.

SNAP-25 is a core component of the SNARE complex, essential for the fusion of synaptic vesicles with the presynaptic membrane, neurotransmitter release, and vesicle pool refilling [23, 24]. Located presynaptically at the NMJ [8, 25], SNAP-25 undergoes phosphorylation at threonine 138 (T138) by protein kinase A (PKA) [26, 27] and at serine 187 (S187) by PKC [15, 28, 29]. Specifically, PKCε is specifically responsible for the activity-dependent SNAP-25 (S187) phosphorylation at the NMJ [8]. Furthermore, nerve-induced muscle contraction exerts a retrograde effect on the PKC-induced phosphorylation of Munc18-1 and SNAP-25, modulating their performance in an activity-dependent manner [7, 8]. This retrograde signalling also affects the expression and function of M_1 and M_2 receptors [30], highlighting that presynaptic activity and the resulting muscle contraction are mutually coupled to control the neurotransmission mechanism.

Therefore, although PKC is essential for neurotransmission at the NMJ, the specific molecular signalling pathways linking PKC activation to ACh release in response to presynaptic activity and to nerve-induced muscle contraction remain unclear, particularly regarding their regulation by mAChRs activation. In this study, we aimed to investigate whether the activity-dependent regulation of M_1 and M_2 mAChRs modulates the classical PKCβ1 and novel PKCε isoforms, as well as the phosphorylation of their key downstream targets, Munc18-1 and SNAP-25, which are crucial for ACh release at the NMJ. To analyse this, we investigated the effect of selective muscarinic receptor blockade (M_1 and M_2) during

presynaptic activity and nerve-induced muscle contraction assessing the subsequent activation of PKC isoforms and phosphorylation of their respective targets. Our findings elucidate the molecular mechanism underlying the activity-dependent interaction between the M_1 and M_2 mAChRs pathways and presynaptic PKC isoforms, ensuring a balanced and efficient process of ACh release. Understanding these molecular pathways is particularly relevant in the context of neuromuscular disorders, as they provide valuable insights into potential therapeutic interventions aimed at restoring optimal neuromuscular function.

Methods

Animal care

Male and female Sprague Dawley rats, aged between 30 and 40 days and sourced from Criffa, Barcelona, Spain, were cared for according to the ethical standards mandated by the European Community Council Directive for laboratory animals. To collect tissue samples, the animals were euthanized in a humane manner using a lethal dose of 4% tribromoethanol from Sigma-Aldrich. Each experimental condition comprised at least three animals ($n \geq 3$) to ensure biological replication. All animal procedures were approved by the Ethics Committee of Animal Experimentation at Universitat Rovira i Virgili (reference number of approved project by Generalitat de Catalunya: 10,760, 23.01.2020), which is based on the Basel Declaration Statement and follows the 3R principle (replace, reduce, refine).

Antibodies

For the Western blotting investigations in this study, we obtained primary and secondary antibodies from selected suppliers, with their concentrations outlined in Table 1. Before employing the Western blotting method, we validated the specificity of primary antibodies by detecting specific bands at their anticipated molecular weights, as expected [7–9, 30].

Reagents

To block muscle contraction, we applied μ-conotoxin GIIIB (#C-270, Alomone Labs Ltd, Jerusalem, Israel), known for its ability to inhibit sarcolemmal voltage-dependent sodium channels (VGSCs) while leaving synaptic ACh release and ACh signalling unaffected [31, 32]. The toxin was utilized in a lyophilized powder form with a purity exceeding 99%. We prepared a stock solution of μ-conotoxin GIIIB at a concentration of 150 μM. The working concentration, used 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%), was 1.5 μM. This solution was oxygenated with a mixture of O_2 and CO_2 in a ratio of 95:5.

Table 1 Antibodies

Target	Epitope	Source	Company (#cat)	Dilution
mAChR M_1	Hu M1 mAChR residues within 227–353 (3rd intracellular loop)	Rb pAb	Alomone Labs (AMR-001)	1/1000
mAChR M_2	Hu M2 mAChR residues within 225–356 (3rd intracellular loop)	Rb pAb	Alomone Labs (AMR-002)	1/1000
PKCβ1	Hu PKCβ1 C-terminus	Ms mAb	Santa Cruz (sc-8049)	1/1000
pPKCβ1 (T642)	Hu PKCβ1 residues 640–644	Rb pAb	Abcam (ab5782)	1/1000
PKCε	Hu PCKε C-terminus	Rb pAb	Santa Cruz (sc-214)	1/1000
pPKCε (S729)	Hu PKCε residues around pS729	Rb pAb	Santa Cruz (sc-12355)	1/1000
Munc18-1	Hu Munc18-1 residues around Y157	Rb pAb	CST (13,414)	1/1000 1/500
pMunc18-1 (S313)	Hu Munc18-1 residues 307–319	Rb pAb	Abcam (ab138687)	1/1000
SNAP-25	Hu SNAP-25 residues around Gln116	Rb mAb	CST (5309)	1/1000 1/500
pSNAP-25 (S187)	Rat SNAP-25 residues around pS187	Rb pAb	Abcam (ab169871)	1/1000
S100 (β-Subunit)	Epitope located on the β-chain (i.e., in S-100a and S-100b)	Ms mAb	Sigma-Aldrich (S2532)	1/50
Syntaxin	Rat Syntaxin	Ms mAb	Millipore (S0664)	1/1000
Neurofilament 200	Epitope in the tail domain of neurofilament 200 (also referred to as the H-subunit)	Ms mAb	Sigma-Aldrich (N0142)	1/1000
Secondary antibodies	Anti-Rb conjugated HRP	Dk pAb	Jackson ImmunoResearch (711–035-152)	1/10000
	Anti-Ms conjugated HRP	Rb pAb	Sigma-Aldrich (A9044)	1/10000
	Anti-Ms conjugated TRITC	Dk pAb	Jackson ImmunoResearch (715–025-151)	1/1000

Table 1 (continued)

Target	Epitope	Source	Company (#cat)	Dilution
	Anti-Rb conjugated Alexa fluor 488	Dk pAb	Invitrogen Antibodies (A-21206)	1/1000
	α-Bungarotoxin conjugated Alexa Fluor 647		Thermo Scientific (B35450)	1/800

Antibodies used in this study and procedure specifications

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

To assess the impact of muscarinic inhibition, we used Pirenzepine dihydrochloride (PIR) (#1071, Tocris) as an M_1 mAChR selective antagonist, with a 10 mM stock solution used at 10 µM, and Methoctramine tetrahydrochloride (MET) (#M105, Sigma) as an M_2 mAChR selective antagonist, with a 1 mM stock solution used at 1 µM, as previously described in basal condition without electrical stimulation of hemidiaphragm [6, 33]. Although this concentration is commonly considered selective for M_2, potential off-target effects, including partial blockade of other mAChR subtypes or cellular side effects [34, 35], cannot be completely excluded and were considered during data interpretation. In both control and drug-containing conditions, all chemicals were diluted in Ringer's solution containing 0.1% dimethyl sulfoxide (DMSO) as the vehicle.

Tissue dissection and treatment

The diaphragm muscle, recognized as a reliable model for studying NMJ development and function [36–38], underwent precise dissection to preserve phrenic nerve connections. Following isolation, nerve-muscle preparations were immersed in Ringer's solution and maintained at a steady temperature of 26 °C throughout the experiment.

In this ex vivo experimental setup, one hemidiaphragm was allocated to the experimental treatment, while its counterpart served as an untreated control for comparative analysis. Muscle contraction was induced by stimulating the phrenic nerve at a frequency of 1 Hz, chosen to sustain various tonic functions without depleting synaptic vesicles. Stimulation, lasting 30 min, was delivered using the A-M Systems 2100 isolated pulse generator (A-M System), following established research protocols [2, 7, 8, 25, 30].

We designed a stimulation protocol specifically aimed at sustaining nerve stimulation and its associated neurotransmission mechanisms, thereby preventing non-nerve-induced (direct) muscle contraction mechanisms [39–41]. To confirm muscle contraction, we relied on visual inspection. Our study comprised four distinct experiments, each designed to elucidate the effects of Pirenzepine dihydrochloride (PIR) as an M_1 mAChR selective antagonist, and Methoctramine tetrahydrochloride (MET) as an M_2 mAChR selective antagonist under conditions of synaptic activity or nerve-induced muscle contraction, as demonstrated in Fig. 1.

1) To estimate the effect of M_1 mAChR inhibition by PIR under synaptic activity we compared presynaptically stimulated muscles whose contraction was blocked by µ-CgTx-GIIIB with and without PIR: ES *vs* ES + PIR.
2) To demonstrate the influence of M_2 mAChR inhibition by MET during synaptic activity, we compared muscles stimulated presynaptically, with contraction blocked by µ-CgTx-GIIIB, both with and without MET: ES *vs* ES + MET.
3) To show the impact of the PIR under muscle contraction, we compared stimulating and contracting muscles with and without PIR: (ES + C) *vs* (ES + C) + PIR.
4) To demonstrate the effects of M_2 mAChR inhibition during muscle contraction, we compared muscles that were stimulated and contracted with and without MET: (ES + C) *vs* (ES + C) + MET.

For experiments requiring stimulation without muscle contraction, we introduced µ-CgTx-GIIIB (refer to the Reagents section). Prior to the application of µ-CgTx-GIIIB, we conducted a thorough visual examination to confirm the accuracy of muscle contraction [2, 7, 8, 25, 30].

Sample processing by Western blotting and fractionation

After concluding the treatment, we immediately froze the whole cell lysate samples for preservation. For a more in-depth understanding of the homogenization and Western blotting procedures utilized, we suggest consulting our previous research Cilleros-Mañé et al., 2021 [9].

Band densitometry analysis was performed utilizing ImageJ 1.52a software (National Institutes of Health, USA) [42]. The integrated optical density of the bands underwent normalization against both background values and the total protein transferred onto PVDF membranes. Total protein analysis was conducted using Sypro Ruby protein blot stain from Bio-Rad [43].

Relative differences between the experimental and control samples were assessed using the same membrane image. The findings presented in this study are based on densitometry measurements obtained from 3–10 individual replicates, with comparisons made against control samples. Importantly, the data quantification was performed in a blinded manner.

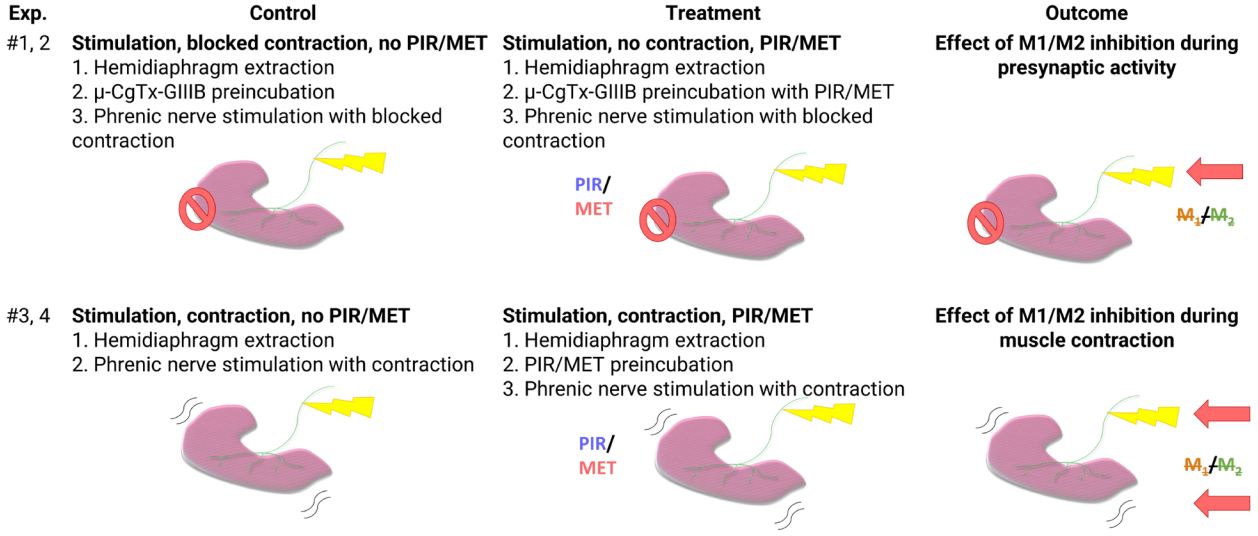

Fig. 1 Design of experimental treatment for the study of effects of M_1 or M_2 mAChRs inhibition during presynaptic activity and nerve-induced muscle contraction. μ-CgTx-GIIIB, μ-conotoxin GIIIB; PIR, Pirenzepine dihydrochloride (M_1 mAChR selective antagonist); MET, Methoctramine tetrahydrochloride (M_2 mAChR selective antagonist)

Immunohistochemistry

To validate the presynaptic specificity of the signalling components analysed by Western blot, we conducted immunohistochemical (IHC) localization of Munc18-1 and SNAP-25 in the *levator auris longus* (LAL) muscle. These proteins were chosen as presynaptic markers to confirm the presynaptic localization of the molecular mechanisms previously described in the same biological context used for biochemical studies. We used the protocol detailed by Cilleros-Mañé et al., 2021 [9]. The LAL muscle samples for this analysis were taken from the same animals ($n = 3$) that were used in our Western blot experiments. Immunohistochemistry was performed on the LAL muscle due to its thinness, which facilitates high-resolution imaging of NMJs, and because previous studies have shown that LAL and diaphragm muscles yield consistent and comparable results in terms of synaptic labelling (e.g., [2, 5, 9, 44]). Western blot analyses were carried out on diaphragm samples subjected to treatment protocols to ensure consistent biochemical quantification while also reducing the number of animals used, in accordance with ethical guidelines concerning the reduction of animal use.

As part of our control procedures, we processed some muscle samples through immunohistochemistry without adding primary antibodies. These control samples exhibited no positive staining. In experiments involving double-staining, omitting either primary antibody resulted in the absence of the respective staining, with no cross-reactivity detected between the remaining primary antibody. To ensure validity of our results, we included at least three muscle samples as negative controls in our experimental setup.

To examine immunolabeled NMJs in whole-mount muscles, we used a Zeiss LSM880 AiryScan confocal microscope paired with a Zeiss PlanApo Å ~ 63 1.42 NA oil objective [9]. For quantitative colocalization analysis, we used the Pearson correlation coefficient (r) to measure the association between two probes within an image and conducted 3D colocalization analyses on confocal stacks using FIJI (ImageJ 1.54f) software (National Institutes of Health, USA) [45]. Representative images were assembled using Adobe Photoshop 8.0.1 (Adobe Systems, San Jose, CA).

Statistical analysis

In all the experiments described, we ensured a minimum of three animals ($n \geq 3$) were used as biological replicates. Each experiment was conducted with at least three biological replicates, and each biological replicate was evaluated in three technical replicates. The sample size determination, aimed at optimizing animal use, was based on criteria established in prior studies [46, 47].

The results, expressed as mean ± SEM (Standard Error of the Mean), are displayed as ratios or percentages relative to the control. Sample normality was assessed using the Shapiro–Wilk test, and statistical differences were determined using either the paired Student's t-test or the non-parametric Wilcoxon test. All analyses were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, Boston, Massachusetts, USA).

In the figures, each dot within the bars represents the mean result obtained from an individual animal. Statistical significance was indicated using the following thresholds: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

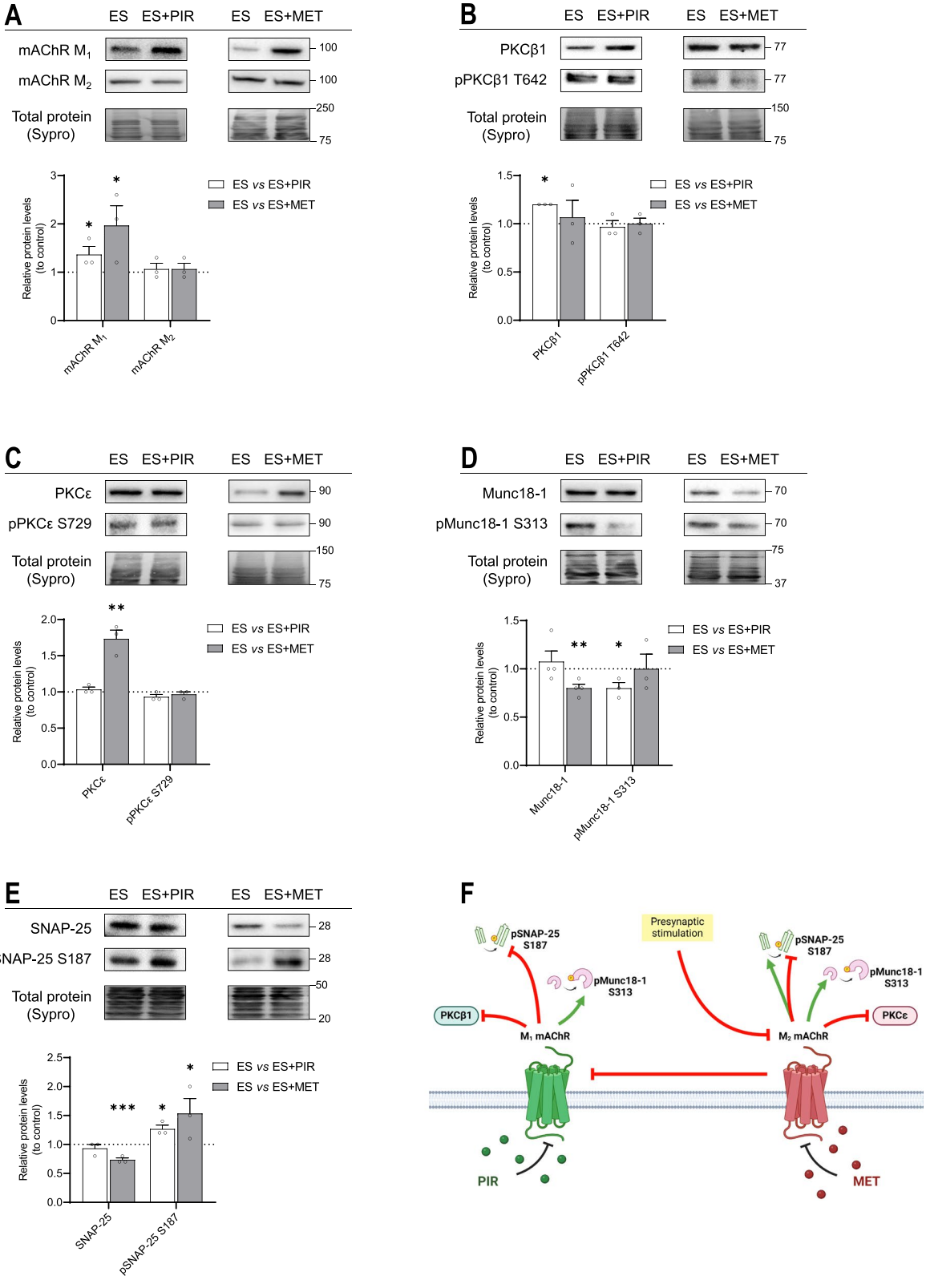

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Modulation by PIR or MET of M_1 and M_2 mAChRs, different presynaptic isoforms of PKC and their targets Munc18-1 and SNAP-25 protein levels during presynaptic activity. **A-D** Western blot analysis of protein levels after treatment with presynaptic stimulation with PIR —ES versus ES + PIR— and with MET —ES versus ES + MET. **A** M_1 and M_2 mAChRs. **B** PKCβ1 and its phosphorylated form pPKCβ1 T642. **C** PKCε and its phosphorylated form pPKCε S729. **D** Munc18-1 and its phosphorylated form pMunc18-1 S313. **E** SNAP-25 [30] and its phosphorylated form pSNAP-25 S187. Data are expressed relative to experimental control – ES (dotted line) (mean ± SEM). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the corresponding control. **F** Graphical representation of the results, the effect of PIR and MET during presynaptic stimulation on presynaptic PKC isoforms and targets. ES, electrical stimulation; M_1/M_2 mAChR, muscarinic acetylcholine receptor M_1/M_2; PKCβ1/PKCε, protein kinase C β1/ε; Munc18-1, mammalian homologue of UNC-18; SNAP-25, synaptosome-associated protein 25; PIR, Pirenzepine dihydrochloride (M_1 mAChR selective antagonist); MET, Methoctramine tetrahydrochloride (M_2 mAChR selective antagonist)

Results

Presynaptic stimulation regulates muscarinic pathway and muscarinic-induced PKC-dependent phosphorylation of Munc18-1 and SNAP-25

To analyse how the nerve-induced activity controls muscarinic receptors to regulate synaptic pathways governing neurotransmission at the NMJ, we applied presynaptic electrical stimulation (ES) together with Pirenzepine dihydrochloride (PIR), M_1 mAChR selective antagonist (ES + PIR), or Methoctramine tetrahydrochloride (MET), M_2 mAChR selective antagonist (ES + MET), and then analysed several key members of the neurotransmission pathway, including muscarinic receptors, PKC isoforms and their downstream targets. The addition of PIR during presynaptic stimulation (ES *vs* ES + PIR) increased the level of M_1 mAChR, while M_2 mAChR levels remained unchanged. Similarly, the addition of MET during presynaptic stimulation (ES *vs* ES + MET) also led to an upregulation of M_1 mAChR, with no significant effect on M_2 mAChR levels (Fig. 2A). The amount of PKCβ1 was increased during presynaptic stimulation with PIR but did not change with MET. However, blocking M_1 or M_2 respectively with PIR or MET in the presence of nerve stimulation did not affect pPKCβ1 T642 (Fig. 2B). Oppositely, nPKCε remained unchanged during presynaptic stimulation with PIR and was upregulated in the same condition with MET. pPKCε S729 was not affected by any of the selective antagonists (Fig. 2C). Together, the results show that the phosphorylation of the presynaptic PKC isoforms is independent of the muscarinic receptors when the nerve-induced activity is present.

Regarding the targets of PKC, Munc18-1 (Fig. 2D) was unaffected by PIR but was downregulated by MET. At the same time, pMunc18-1 S313 was decreased in response to presynaptic stimulation with PIR but did not change with MET. SNAP-25 (Fig. 2E) as Munc18-1 was downregulated only by MET but not by PIR. However, pSNAP-25 S187 was upregulated by both MET and PIR during presynaptic activity.

In summary, during nerve-induced presynaptic activity, the M_1 mAChR or M_2 mAChR blockage induced an accumulation of the M_1 mAChR. In the same presynaptic stimulation condition, M_1 mAChR downregulated PKCβ1, while M_2 mAChR decreased the level of PKCε. Additionally, M_2 mAChR upregulated the total levels of both targets of PKC, Munc18-1 and SNAP-25, but downregulated pSNAP-25 S187. In contrast, M_1 mAChR oppositely affected phosphorylated forms of Munc18-1 and SNAP-25, upregulating pMunc18-1 S313 and downregulating pSNAP-25 S187. Figure 2F shows a graphical summary of these findings.

To assess the presynaptic localization of SNAP-25 and Munc18-1 at the NMJ, we performed immunohistochemical labelling and quantitative colocalization analysis (Fig. 3). Munc18-1 showed a strong colocalization with the presynaptic markers Syntaxin and Neurofilament (Synt + NF) ($r = 0.82 \pm 0.01$), confirming its enrichment within the presynaptic terminal [7, 9]. In contrast, its overlap with postsynaptic AChRs was moderate ($r = 0.53 \pm 0.02$), indicating a clear spatial separation from the postsynaptic domain.

SNAP-25 followed a similar distribution pattern, displaying high colocalization with Synt + NF ($r = 0.72 \pm 0.02$), and a moderate correlation with AChRs ($r = 0.58 \pm 0.01$). Together, these findings support the selective presynaptic localization of both proteins and underscore their muscarinic-induced roles in synaptic vesicle dynamics within motor neuron terminals [7, 9, 25, 30].

Nerve-induced muscle contraction regulates muscarinic pathway and muscarinic-induced PKC-dependent phosphorylation of Munc18-1 and SNAP-25

In the muscle contraction condition, M_1 mAChR was upregulated with both selective antagonists PIR ((ES + C) vs (ES + C) + PIR) and MET ((ES + C) vs (ES + C) + MET), while M_2 mAChR was unaffected by any of them (Fig. 4A).

Regarding presynaptic PKCs, the total protein level of PKCβ1 and its phosphorylated form, pPKCβ1 T642, were increased during nerve-induced muscle contraction with PIR but were unaffected by MET (Fig. 4B). PKCε and its phosphorylated form, pPKCε S729, were regulated differently by PIR and MET during muscle contraction. M_1 mAChR blockage by PIR upregulated pPKCε S729 but did not change the total levels of PKCε. In contrast, M_2 mAChR blockage by MET during nerve-induced muscle contraction increased the level of PKCε but downregulated pPKCε S729 (Fig. 4C).

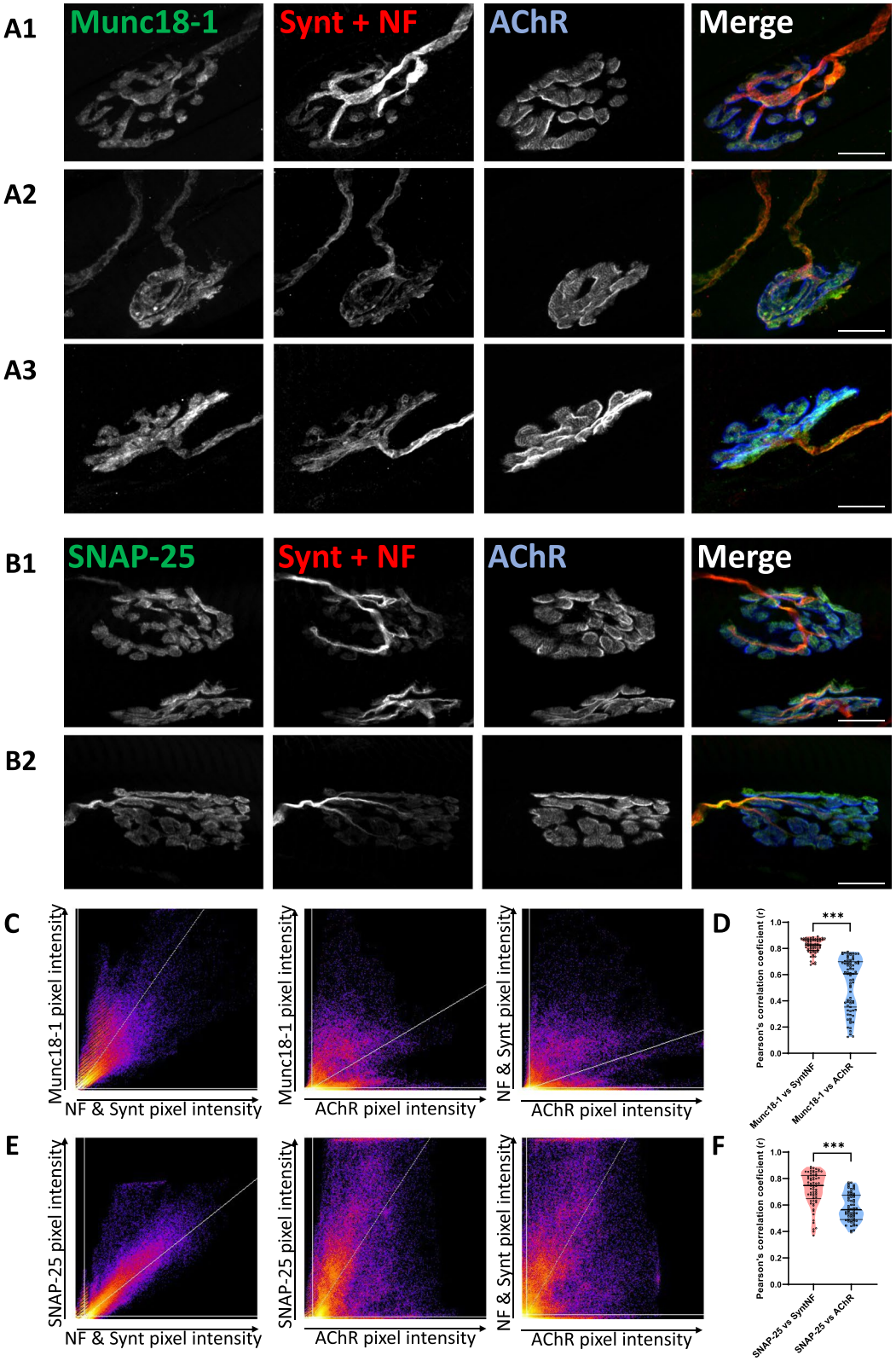

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Munc18-1 and SNAP-25 localization at the adult NMJ. **A1-A3** Super resolution image of a NMJ with triple labelling: Munc18-1 (green), Neurofilament + Syntaxin (red), and AChR (blue). **B1-B2** Confocal image of a NMJ with triple labelling: SNAP-25 (green), Neurofilament + Syntaxin (red), and AChR (blue). Scale bar: 10 µm. **C** Example of quantitative colocalization represented as heatmap of the intensity of between anti-Munc18-1 labelling, presynaptic Neurofilament and Syntaxin labelling and postsynaptic AChR labelling. **D** Average Pearson's correlation coefficient of the colocalization between Munc18-1 versus Neurofilament and Syntaxin and versus AChRs. **E** Example of quantitative colocalization represented as heatmap of the intensity of between anti-SNAP-25 labelling, presynaptic Neurofilament and Syntaxin labelling and postsynaptic AChR labelling. **F** Average Pearson's correlation coefficient of the colocalization between SNAP-25 versus Neurofilament and Syntaxin and versus AChRs

The targets of these PKC isoforms were differently affected by M_1 mAChR and M_2 mAChR blockage by PIR and MET, respectively. The total Munc18-1 protein level was downregulated by MET, during nerve-induced muscle contraction. However, the phosphorylated form pMunc18-1 S313 remained unchanged by any of the antagonists (Fig. 4D). The levels of total SNAP-25 protein were not affected by both M_1 mAChR and M_2 mAChR blockage. In contrast, pSNAP-25 S187 was upregulated during nerve-induced muscle contraction with both PIR and MET (Fig. 4E).

In summary, during nerve-induced muscle contraction, as occurs with presynaptic activity, both M_1 and M_2 mAChR blockade cause an accumulation of M_1. Thus, in a condition of presynaptic stimulation resulting in contraction or not, M_1 in order to be functional needs M_2 activation to maintain its level and prevent accumulation. M_1 mAChR downregulated PKCβ1 and its phosphorylated form, pPKCβ1 T642, as well as pPKCε S729. On the other hand, M_2 mAChR downregulated PKCε but upregulated pPKCε S729. Regarding the targets, both M_1 and M_2 mAChRs downregulated pSNAP-25 S187 and only M_2 mAChR upregulated Munc18-1, though not its phosphorylated form. Figure 4F shows a graphical summary of these findings.

Discussion

The M_1 and M_2 muscarinic receptor subtypes regulate ACh release at the NMJ, but they perform opposite functions, as M_1 increases it, whereas M_2 decreases the end-plate potential [6, 13]. Although M_1 and M_2 receptors are both present at the NMJ, M_2 is more abundantly expressed and more robustly detected at the NMJ, as previously reported [11, 48–52]. Different modulations by muscarinic receptors control PKC presynaptic isoforms, PKCβ1 and PKCε, to regulate the phosphorylation of key molecules of the exocytotic synaptic vesicle machinery [2, 4, 5, 7–9] to balance neurotransmission [6, 13].

Interestingly, M_1 and M_2 muscarinic receptors [30] and presynaptic PKC isoforms [2, 4, 5] are upregulated by nerve-induced muscle contraction; however, the molecular mechanisms and the potential interaction between these two retrograde regulations have not been studied until now. Here, we provide the mechanism demonstrating the cooperation of muscarinic receptors to regulate presynaptic PKC isoforms and their synaptic targets in response to neural activity at the neuromuscular system. These results enhance our understanding of how functional bidirectional communication is regulated at the NMJ.

In this study, we identified a muscarinic signalling pathway confined to the presynaptic terminal of the NMJ, involving M_1 and M_2 mAChRs, PKCβI, PKCε, and the presynaptic targets Munc18-1 and SNAP-25. This was supported by biochemical fractionation and immunofluorescence analyses showing selective enrichment of these components in the synaptic region and their involvement in neurotransmitter release [2, 5, 7–9, 44, 53]. Therefore, it is expected that the results obtained from the mAChR regulation on these PKC isoforms and their downstream pathways are located in the presynaptic compartment of the NMJ. However, although muscarinic receptors, specifically M_1 and M_2 subtypes, have been identified primarily on presynaptic terminals at the NMJ, playing essential roles in modulating autocrinally synaptic transmission [6, 9, 11, 12, 33, 54], the localization of M_1 and M_2 receptors in perisynaptic glial cells [11, 55, 56], the muscle cells [49, 51, 57–59], and the observed modulation of mostly postsynaptic PKCα by M_2 antagonism [9]s, suggest that muscarinic signalling may not be restricted to the presynaptic terminal. Notably, if some of the mAChR effects start in the perisynaptic glia cells or the postsynaptic cell, this would indicate a level of intercellular communication mediated by muscarinic signalling and underscore the complexity of its regulation within a tripartite synapse. For example, stabilization of nAChRs at the postsynaptic membrane depends on synaptic activity and PKC signalling, particularly involving PKCθ [60, 61], and this process may be influenced by presynaptic mAChR signalling through modulation of ACh release. Altogether, these findings support a model in which mAChRs contribute to coordinated, compartment-spanning signalling events that integrate motoneuronal, glial, and muscle-derived pathways at the NMJ.

Moreover, we conducted immunohistochemical labelling and colocalization analyses for SNAP-25 and Munc18-1. The results confirmed the presynaptic localization of SNAP-25 and Munc18-1 and underscored their functional involvement in muscarinic-mediated synaptic vesicle regulation within motor neuron terminals at the NMJ during synaptic activity [7–9, 25, 30], reinforcing the anatomical specificity of the signalling cascade we describe, referring to the presynaptic nerve terminal. This is especially important given that biochemical analyses

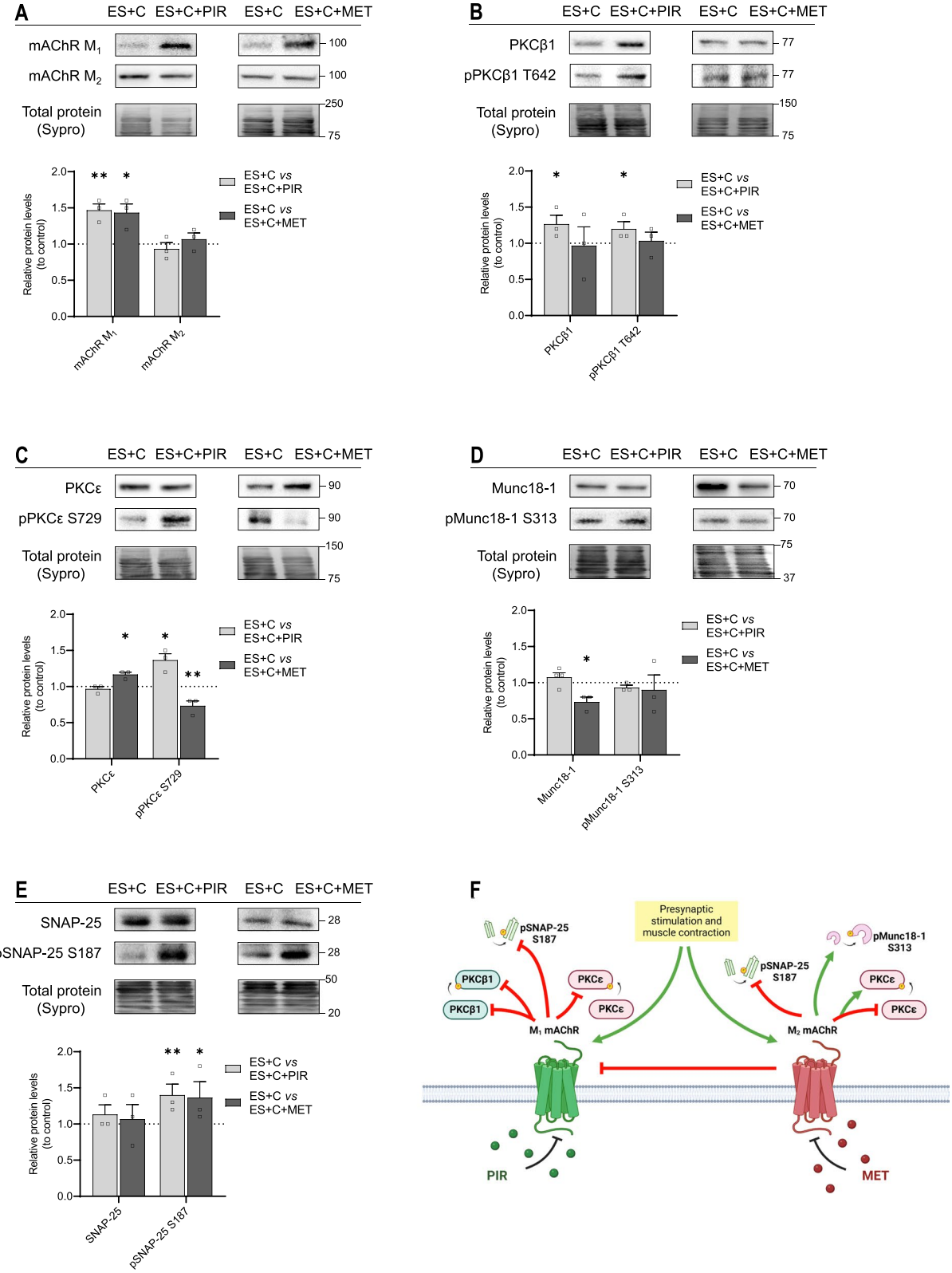

Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 Modulation by PIR or MET of M_1 and M_2 mAChRs, different isoforms of PKC and its targets Munc18-1 and SNAP-25 protein levels during nerve-induced muscle contraction. **A-D** Western blot analysis of protein levels after treatment with presynaptic stimulation and nerve-induced muscle contraction with PIR —ES+C versus ES+C+PIR— and with MET —ES+C versus ES+C+MET. **A** M_1 and M_2 mAChRs. **B** PKCβ1 and its phosphorylated form p PKCβ1 T642. **C** PKCε and its phosphorylated form pPKCε S729. **D** Munc18-1 and its phosphorylated form pMunc18-1 S313. **E** SNAP-25 [30] and its phosphorylated form pSNAP-25 S187. Data are expressed relative to experimental control – ES+C (dotted line) (mean ± SEM). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the corresponding control. **F** Graphical representation of the results, effect of PIR and MET during presynaptic stimulation with nerve-induced muscle contraction on presynaptic PKC isoforms and targets. ES + C, electrical stimulation with muscle contraction; M_1/M_2 mAChR, muscarinic acetylcholine receptor M_1/M_2; PKCβ1/PKCε, protein kinase C β1/ε; Munc18-1, mammalian homologue of UNC-18; SNAP-25, synaptosome-associated protein 25; PIR, Pirenzepine dihydrochloride (M_1 mAChR selective antagonist); MET, Methoctramine tetrahydrochloride (M_2 mAChR selective antagonist)

were performed in whole-muscle extracts, which contain multiple cell types.

M1 mAChR is dependent on M2 mAChR under synaptic activity

Our findings reveal that the activity of M_1 mAChR is dependent on M_2 mAChR, regardless of the synaptic condition, while M_2 operates independently of M_1. This regulatory relationship aligns with previous observations under basal conditions [9, 30], indicating an endogenous regulation of M_2 on M_1 at the NMJ. This interaction also provides a molecular explanation for previous findings demonstrating that M_2 overcomes M_1 functionally at this synapse [9, 13]. The downregulation of M_1 protein levels by M_2 may occur through receptor internalization and degradation [62], or via a decrease of PKA/pCREB-mediated M_1 synthesis [63]. Furthermore, the M_2 mAChR inhibition over M_1 mAChR at the NMJ could interact with the synaptic activity downregulation over M_2 mAChR [30], but it is not strong enough to counter it. These findings suggest that rhythmic stimulation alone may induce changes in mAChR expression, particularly a reduction in M_2 levels, potentially altering synaptic efficacy over time. This dynamic regulation should be considered when designing electrophysiological protocols, as it may impact endplate potential recordings and muscle force measurements during prolonged stimulation. Interestingly, although nerve stimulation reduces M_2 receptor expression, this does not result in a compensatory increase in M_1 receptor levels [30], despite the proposed inhibitory influence of M_2 on M_1 expression [33]. This suggests that M_1 receptor regulation is likely modulated by additional mechanisms beyond M_2-mediated signalling, possibly involving activity-dependent transcriptional control, neurotrophic signalling, or other intracellular pathways that override this effect [64–66]. Alternatively, changes in M_1 expression may be transient or occur post-transcriptionally, and therefore not detected under our experimental conditions. Additionally, we observed that pharmacological blockade of M_1 mAChRs, regardless of the synaptic activity conditions, results in increased M_1 protein levels. This indicates that M_1 activity is required to maintain its own expression levels, potentially through an activity-dependent regulation of internalization and degradation [62]. When M_1 is inactive, these degradation pathways may be reduced, leading to the accumulation of M_1. In parallel, the PKA/pCREB-induced synthesis [63] could be continuously contributing to the sustained increase in M_1 expression.

Our results reveal a unidirectional regulatory relationship in which M_2 mAChRs modulate M_1 receptor expression and activity at the NMJ, independently of synaptic stimulation. This cross-talk provides a molecular basis for the dominant role of M_2 in cholinergic signalling and underscores the importance of receptor interaction in maintaining synaptic balance, ensuring synaptic stability and preventing overstimulation. The dynamic interplay between receptor activity, internalization, degradation, and synthesis underscores the complexity of muscarinic receptor regulation and suggests that alterations in this balance could have functional consequences for synaptic transmission and plasticity. These insights contribute to a deeper understanding of receptor cross-talk in peripheral synapses and may have implications for targeting mAChRs in neuromuscular disorders or cholinergic dysfunction.

Presynaptic stimulation regulates muscarinic pathway and muscarinic-induced PKC-dependent phosphorylation of Munc18-1 and SNAP-25

Under conditions of presynaptic activity, M_1 blockade with pirenzepine (PIR) increased PKCβ1 protein amount without affecting PKCβ1 T642 phosphorylation, which could indicate an increase in the synthesis of PKCβ1 and/or an accumulation of the kinase. Therefore, M_1 mAChR downregulates PKCβ1 but does not affect the priming of the kinase that remains unaltered. Several reasons indicate that M_1 might induce PKCβ1 degradation after activation rather than inhibiting PKCβ1 synthesis. First, M_1 is associated with the Gq protein and PKC activation, which leads to PKC activity-dependent degradation [67–72]. Supporting this, we previously found at the NMJ that PKCβ1 undergoes degradation after phrenic nerve stimulation to potentiate ACh release [2, 53]. Further evidence indicates that M_1 mAChR triggers PKCβ1 activity and its turnover through membrane-associated pathways. PKC downregulation in basal conditions occurs at the membrane fraction [9], consistent with studies showing, in cell cultures, activity-dependent PKCβ1 ubiquitination and degradation at the membrane [69]. Additionally, M_1

mAChR requires PKCβ1 activity to induce phosphorylation of Munc18-1 at S313 under basal conditions [9], a modification associated with SNARE complex regulation and enhanced neurotransmission [19]. Our current results extend these findings, showing that M_1 increases the nerve-induced pMunc18-1 S313 levels, reinforcing its role in PKC-dependent modulation of neurotransmitter release. This is in accordance with a PKC activity-dependent function and subsequent degradation. Together, these results add to our knowledge the insights about the mechanism of M_1 enhancement and regulations of acetylcholine release at the NMJ [13]. In contrast, total SNAP-25 protein levels were unaffected by M_1 mAChR, while its phosphorylation at S187 was downregulated upon M_1 activity. This could indicate that M_1 may influence the PKCε pathway, as PKCε is associated with pSNAP-25 S187 phosphorylation [8]. However, unlike PKCβ1, PKCε protein levels and its phosphorylation were not altered by M1 blockade. This distinct behaviour between PKCβ1 and PKCε could occur because PKCε is less sensitive to activity-dependent degradation. Indeed, PKCε rate of downregulation is threefold slower than other PKC isoforms [73].

M_2 mAChR blockade with MET did not affect PKCβ1 expression or phosphorylation, nor Munc18-1 phosphorylation, further highlighting the selective relationship between PKCβ1 and M_1 signalling. Therefore, M_2 signalling does not affect the classical PKCβ1 isoform priming or levels, nor its catalytic activity on Munc18-1. In basal conditions, M_2 mAChR downregulates PKCε synthesis, potentially through the inhibition of PKA activity [33]. During presynaptic activity condition, M2 signalling likewise downregulates total PKCε protein levels without affecting its phosphorylated form and also reduces pSNAP-25 S187, suggesting that M_2 modulates the PKCε–SNAP-25 pathway and indicating that the decrease in the PKCε total protein levels could affect the amount of its phosphorylated targets like SNAP-25 [9].

Our findings also suggest that muscarinic receptor activity could regulate synaptic protein levels, including SNAP-25, Munc18-1, and the receptors themselves, via three interconnected mechanisms. First, M_1 and M_2 receptors modulate kinase cascades (PKC/PKA), influencing transcription factors such as CREB and altering protein synthesis [30, 33, 74–76]. Second, receptor internalisation and trafficking affect both receptor turnover and the stability of associated protein complexes [77–80]. Third, mAChRs impact the mTOR pathway, which governs global protein synthesis and degradation [68, 74, 81–88]. Together, these mechanisms provide a flexible regulatory framework that links synaptic activity to protein abundance and receptor homeostasis. Interestingly, M_2 mAChR activation under presynaptic condition promoted the synthesis of both SNAP-25 and Munc18-1, indicating a broader regulatory role in maintaining presynaptic protein levels. This suggests that M_2 receptor function is not limited to modulating neurotransmitter release directly but may also influence the structural and functional integrity of the presynaptic terminal. In line with this broader function, Liu and colleagues [89] reported that inhibition of M_2 muscarinic receptors in the medial prefrontal cortex using methoctramine led to increased levels of both BDNF and mTORC1, which is involved in the regulation of protein synthesis [90–94], suggesting that a similar process may operate at the NMJ. Collectively, these mechanisms may contribute to the coordinated regulation of synaptic protein synthesis under conditions of neural activity.

These findings highlight distinct roles for presynaptic-induced M_1 and M_2 mAChRs pathways in modulating PKC signalling pathways that control the phosphorylation of key presynaptic proteins involved in neurotransmitter release at the NMJ (Fig. 5). M_1 mAChR selectively regulates PKCβ1 and drives activity-dependent phosphorylation of Munc18-1, while M_2 influences PKCε levels and modulates SNAP-25 phosphorylation. The differential regulation of PKC isoforms and their targets underscores a finely tuned muscarinic control over synaptic machinery, offering mechanistic insight into how cholinergic signalling dynamically shapes synaptic strength. This layered modulation may be critical for maintaining synaptic efficacy during sustained activity and could inform therapeutic strategies targeting presynaptic dysfunction in neuromuscular disorders.

Nerve-induced muscle contraction regulates muscarinic pathway and muscarinic-induced PKC-dependent phosphorylation of Munc18-1 and SNAP-25

During nerve-induced muscle contraction, M_1 mAChRs blockade with pirenzepine (PIR) increased PKCβ1 protein levels, similar to what we previously observed under presynaptic stimulation conditions without contraction, showing that M_1 mAChRs downregulate PKCβ1 levels. This phenomenon was also observed under basal conditions [33]. These consistent findings support the hypothesis that M_1 mAChRs downregulate PKCβ1 levels across different activity states. Given that TrkB signalling enhances PKCβ1 synthesis during muscle contraction [2], the present data point to a regulatory balance between TrkB-mediated upregulation and M_1 mAChR-mediated downregulation, with TrkB exerting a stronger influence, considering that the net effect during nerve-induced muscle contraction is an increase in PKCβ1 levels [2]. Conversely, M_2 blockade with MET did not alter PKCβ1 levels, indicating its expression is independent of M_2 mAChR activity. Additionally, pPKCβ1 T642 was also upregulated during nerve-induced muscle contraction with M_1 mAChR blockade with PIR. This suggests

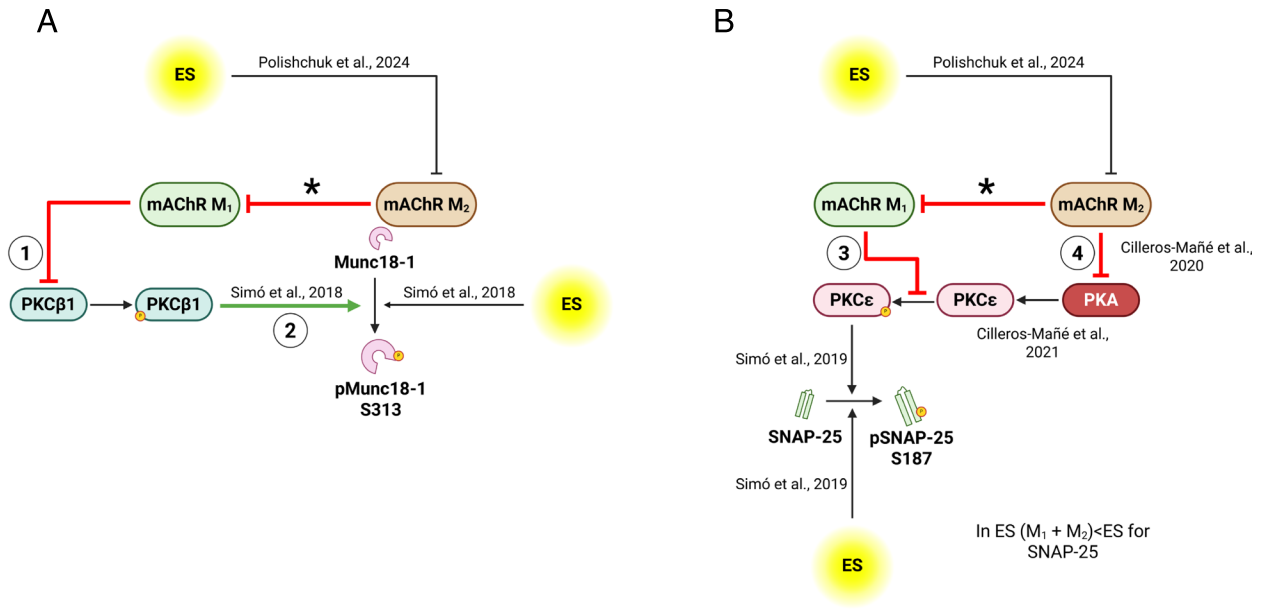

Fig. 5 Graphical representation of the main discovered effects during presynaptic stimulation on presynaptic PKC isoforms and targets. **A** M_1 mAChRs-PKCβ1 pathway and its target Munc18-1. **B** M_2 mAChRs-PKCε pathway and its target SNAP-25. *The main effects:* * – M_2 mAChRs downregulates M_1 mAChRs; 1 – activity-dependent consumption of PKCβ1; 2 – M_1 mAChRs promotes Munc18-1 phosphorylation at S313; 3 – M_1 mAChRs modulates the activity of M_2 mAChRs-PKCε pathway; 4 – possible pathway of downregulation of protein expression. ES, electrical stimulation; M_1/M_2 mAChR, muscarinic acetylcholine receptor M_1/M_2; PKCβ1/PKCε, protein kinase C β1/ε; Munc18-1, mammalian homologue of UNC-18; SNAP-25, synaptosome-associated protein 25

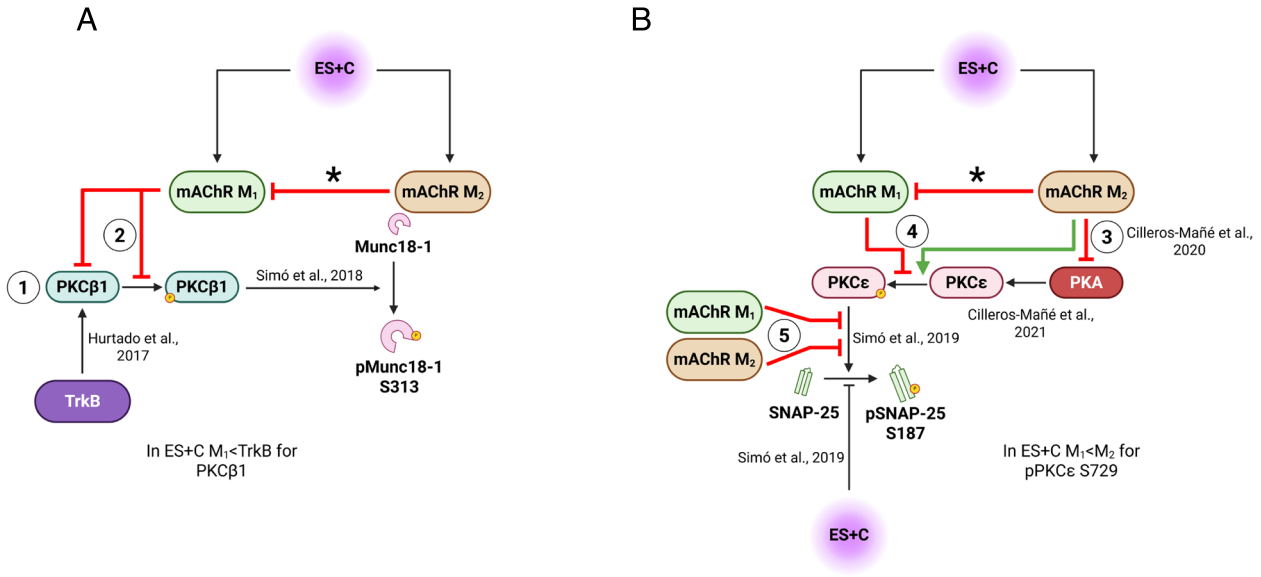

Fig. 6 Graphical representation of the main discovered effects during nerve-induced muscle contraction on presynaptic PKC isoforms and targets. **A** M_1 mAChRs-PKCβ1 pathway and its target Munc18-1. **B** M_2 mAChRs-PKCε pathway and its target SNAP-25. *The main effects:* * – M_2 mAChRs downregulates M_1 mAChRs; 1 – M_1 mAChR downregulates and TrkB upregulates PKCβ1, the effect of TrkB is stronger; 2 – decrease of pPKCβ1 T642 levels due to total protein PKCβ1 decrease; 3 – possible pathway of downregulation of protein expression; 4 – opposite effects of M_1 and M_2 mAChR on PKCε phosphorylation, the effect of M_2 mAChR is stronger; 5 – M_1 and M_2 mAChR downregulate pSNAP-25 S187 phosphorylation. ES + C, electrical stimulation with muscle contraction; M_1/M_2 mAChR, muscarinic acetylcholine receptor M_1/M_2; PKCβ1/PKCε, protein kinase C β1/ε; Munc18-1, mammalian homologue of UNC-18; SNAP-25, synaptosome-associated protein 25

that M_1 mAChR downregulates the phosphorylation of PKCβ1 at T642, negatively regulating PKCβ1 priming. However, muscle contraction alone also elevates pPKCβ1 T642 levels [2], confirming that the contraction's effect via the TrkB pathway outweighs that of M_1 mAChR. The blockade of M_1 during nerve-induced muscle contraction leads to increased accumulation of pPKCβ1 T642, likely due to the inhibition of M_1-mediated downregulation.

Notably, this differs from basal conditions, where PIR reduces pPKCβ1 T642 despite increasing total PKCβ1 levels [9] highlighting a specific effect of presynaptic stimulation on the system.

Phosphorylation of pMunc18-1 at S313 was not altered by M_1 or M_2 blockade during nerve-induced muscle contraction, likely due to the balancing effects of contraction, which has been shown to reduce pMunc18-1 S313 despite nerve stimulation increasing it [7]. Overall, our previous findings indicated that PKCβ1 is essential for the nerve stimulation-induced increase in Munc18-1 phosphorylation at S313, whereas muscle contraction contributes to decreased phosphorylation of Munc18-1 at this site. Therefore, we conclude that nerve-induced muscle contraction balances Munc18-1 phosphorylation [7], which is not dependent on muscarinic receptor activity, as evidenced by the lack of changes in pMunc18-1 protein levels when M_1 and M_2 are blocked. However, inhibition of M_2 mAChR during nerve-induced muscle contraction resulted in downregulation of Munc18-1 protein levels without impacting its phosphorylated form, suggesting that the M_2 mAChR pathway enhances Munc18-1 levels like in presynaptic conditions.

Furthermore, during nerve-induced muscle contraction, M_2 mAChR downregulates PKCε synthesis, potentially through the inhibition of PKA activity [33]. The levels of pPKCε S729 increased with PIR treatment and decreased with MET during concurrent stimulation and muscle contraction, concluding that M_1 mAChR downregulates and M_2 mAChR upregulates phosphorylation of PKCε at S729.

The preservation of SNAP-25 levels, which were downregulated during presynaptic activity, in stimulated muscles undergoing contraction, despite M_2 blockade, suggests that contraction-induced retrograde signals may compensate for the negative regulatory effects of M_2 on presynaptic proteins. Neurotrophic factors released during muscle activity, such as BDNF, are known to enhance presynaptic function and protein expression through TrkB-dependent mechanisms [2, 7, 30, 95–99], which may help maintain SNAP-25 under these conditions. Finally, both M_1 and M_2 mAChRs contribute to the downregulation of pSNAP-25 at S187 during nerve-induced muscle contraction. Since pSNAP-25 S187 is mediated by PKCε [8], its reduction may be related to the influence of M_1 and M_2 mAChRs on PKCε activity. Specifically, M_1's downregulation of pPKCε S729 decreases its activity and, consequently, the levels of pSNAP-25 S187. Simultaneously, M_2 mAChR downregulates PKCε synthesis while upregulating pPKCε S729. Due to reduced PKCε synthesis, the available pPKCε S729 is primarily utilized for pSNAP-25 S187 phosphorylation, resulting in an overall decrease in pSNAP-25 S187 levels. Interestingly, our findings reveal a differential muscarinic regulation of SNAP-25 phosphorylation at two distinct residues: S187 and T138, which may reflect functionally divergent roles in exocytotic control. Phosphorylation at S187, a canonical PKC target [8, 15, 28, 29], has been associated with increased SNARE complex assembly and enhanced vesicle fusion probability, particularly during sustained synaptic activity or neuromodulatory input [8]. Consistent with this, all experimental conditions involving PIR and MET, both with and without muscle contraction, led to increased pSNAP-25 S187 levels, supporting a convergent mechanism for synaptic facilitation through this pathway. In contrast, phosphorylation at T138, mediated by PKA, has been implicated in inhibitory modulation of exocytosis [26, 27, 100]. The lack of change observed in pSNAP-25 T138 levels with PIR, versus the significant reduction observed with MET [30], suggests that M_2 may decrease neurotransmitter release [6, 13] not only by downregulating pSNAP-25 S187 levels but also by increasing the inhibitory effect imposed by pSNAP-25 T138. This dual modulation could represent a more potent mechanism to control of transmitter release under conditions of high demand or plasticity. Together, these observations support the notion that the phosphorylation state of SNAP-25 is tightly regulated through site-specific mechanisms that act in a complementary and potentially hierarchical manner to maintain synaptic efficacy and prevent exhaustion.

Our findings reveal that nerve-induced muscle contraction impacts M_1 and M_2 mAChRs to differentially regulate PKC isoforms and SNARE-related proteins (Fig. 6). M_1 mAChR primarily modulates both PKCβ1 pathway with its target Munc18-1 and PKCε pathway extending effect on its target pSNAP-25 S187, while M_2 influences the synthesis of Munc18-1 and synthesis and regulation of PKCε and its following target – pSNAP-25 S187. During nerve-induced muscle contraction, TrkB signalling and muscarinic receptor activity converge to fine-tune PKC activation and SNARE protein modulation, ensuring balanced neurotransmitter release [2, 7]. This coordinated regulation underscores the role of muscarinic signalling in maintaining synaptic efficacy under physiological demand.

Conclusion

We demonstrate that presynaptic stimulation and nerve-induced muscle contraction differentially regulate M_1 and M_2 mAChRs signalling pathways at the NMJ. Presynaptic stimulation selectively downregulates M_2 expression, while muscle contraction upregulates both M_1 and M_2 receptors. Despite these contrasting effects, M_1 activity remains consistently suppressed by M_2 signalling in both conditions, underscoring a dominant regulatory interaction mechanism. In terms of PKC signalling, we found that M_1 specifically downregulates PKCβ1, whereas M_2

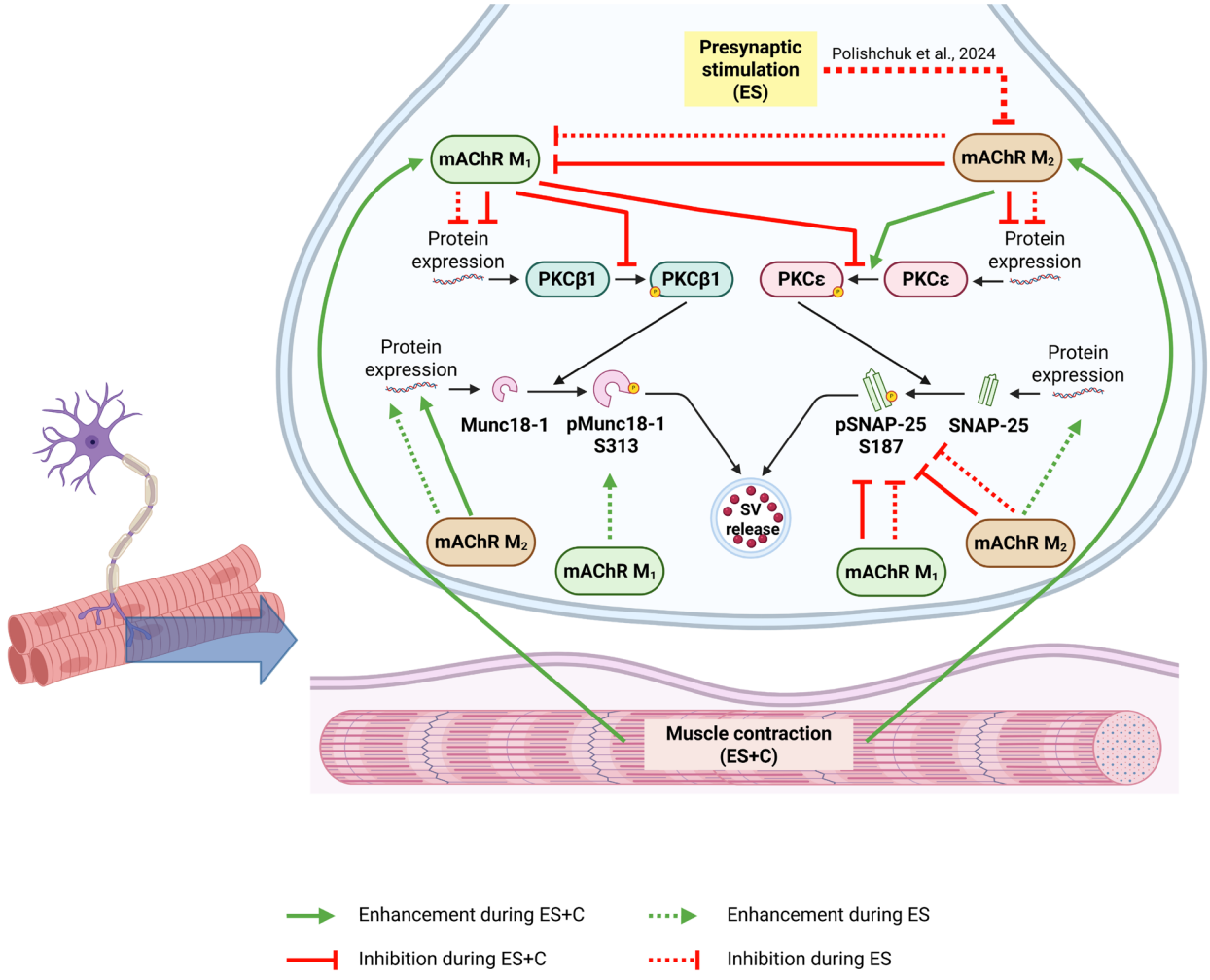

Fig. 7 Summary of muscarinic signalling in regulating the presynaptic PKC pathway and its targets at the NMJ during presynaptic activity and nerve-induced muscle contraction. Model of the neurotransmission machinery regulation resulted from this study during presynaptic activity and nerve-induced muscle contraction

downregulates PKCε in response to both presynaptic and muscle-derived activity. As the retrograde effect of nerve-induced muscle contraction, M_1 downregulates pPKCβ1 T642 and pPKCε S729, while M_2 promotes the accumulation of pPKCε S729.

These regulations over the kinases extend to key downstream effectors, Munc18-1 and SNAP-25, which collectively regulate synaptic vesicle fusion and neurotransmitter release. The M_1-PKCβ1 pathway administrates the phosphorylation of Munc18-1 at S313, thereby influencing synaptic vesicle fusion, while the M_2-PKCε signalling, modulated by M_1 activity during the nerve-induced muscle contraction, downregulates the phosphorylation of SNAP-25 at S187 during both pre- and postsynaptic activities, ultimately affecting neurotransmitter release.

Although our data and previous findings support a prominent role for the PKC pathway, particularly via M_1 and M_2 muscarinic receptor activation, in the modulation of synaptic function at the NMJ, it is important to acknowledge that muscarinic signalling involves multiple intracellular mechanisms. These include, among others, PKA activation, phospholipid depletion, ion channel modulation, and endocannabinoid production [33, 101, 102]. Therefore, the regulation of neurotransmitter release by muscarinic receptors likely results from the integrated action of several pathways, rather than being solely dependent on PKC signalling.

Altogether, our findings uncover a finely tuned molecular mechanism of the activity-dependent communication between M_1 and M_2 mAChR pathways, highlighting their role in balancing the optimal process of ACh release, with M_1 enhancing ACh release and M_2 decreasing it.

A summary of the main findings is represented in Fig. 7.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-025-02440-4.

Supplementary Material 1

Acknowledgements
We thank Dr. Vincent Schram and Dr. Ling Yi in the NICHD Microscopy and Imaging Core (NIH, Bethesda, USA) for assistance with confocal imaging. We would like to express our heartfelt gratitude to Dr. Neus Garcia, whose contribution played a vital role in developing our research. She will always be remembered and missed. This work is dedicated to her memory.

Authors' contributions
AP, VCM, MBS, LJB, CSS, MJEH and MDW: data collection, quantitative analysis, literature search, data interpretation; AP, VCM and LJB: graphic design; AP, VCM, MT, MAL: statistics; AP, JT, EH, and MAL: conception and design, literature search, data interpretation, manuscript preparation. EH, JT, and MAL contributed equally to this work.

Funding
Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This work was supported by funding supported by the Catalan Government (2021SGR01214) and by MCIN/AEI/https://doi.org/10.13039/501100011033/by "ERDF A way of making Europe" (PID2019-106332 GB-I00 and PID2022-141252NB-I00). MBS has been supported by Research Grant (FI, 2021-FI-B00755 Agència de Gestió d'Ajuts Universitat i Recerca (AGAUR)), A.P. by the Spanish Ministerio de Ciencia Innovación y Universidades (grant no. PRE2020-092084, project no. PID2019-106332 GB-I00), V.C.M. by a research contract from the project PID2019-106332 GB-I00 and M.J.E.H. by the Universitat Rovira i Virgili (URV) under the framework of the "Programa Martí i Franquès d'ajuts a la investigació. Contractes de personal investigador predoctoral en formació (2023PMF-PIPF-15)".

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The rats were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All experiments on animals have been reviewed and approved by the Animal Research Ethics Committee of the Universitat Rovira i Virgili (reference number of approved project by Generalitat de Catalunya: 10760, 23.01.2020), which is based on the Basel Declaration Statement and follows the 3R principle (replace, reduce, refine).

Consent for publication
Not applicable for that section.

Competing interests
The authors declare no competing interests.

Author details
[1]Universitat Rovira i Virgili, Unitat d'Histologia i Neurobiologia (UHNeurob), Facultat de Medicina i Ciències de la Salut, C/Sant Llorenç 21, 43201 Reus, Spain
[2]Institut d'Investigació Sanitària Pere Virgili (IISPV), Unitat d'Histologia i Neurobiologia (UHNeurob), Reus, Spain

Received: 20 May 2025 / Accepted: 13 September 2025
Published online: 14 October 2025

References

1. Lanuza MA, Santafe MM, Garcia N, Besalduch N, Tomàs M, Obis T, et al. Protein kinase C isoforms at the neuromuscular junction: localization and specific roles in neurotransmission and development. J Anat. 2014. https://doi.org/10.1111/joa.12106.
2. Hurtado E, Cilleros V, Nadal L, Simó A, Obis T, Garcia N, et al. Muscle contraction regulates BDNF/TrkB signaling to modulate synaptic function through presynaptic cPKCα and cPKCβI. Front Mol Neurosci. 2017;10:1–22.
3. Tomàs JM, Garcia N, Lanuza MA, Nadal L, Tomàs M, Hurtado E, et al. Membrane receptor-induced changes of the protein kinases A and C activity may play a leading role in promoting developmental synapse elimination at the neuromuscular junction. Front Mol Neurosci. 2017;10:255.
4. Obis T, Hurtado E, Nadal L, Tomàs M, Priego M, Simon A, et al. The novel protein kinase C epsilon isoform modulates acetylcholine release in the rat neuromuscular junction. Mol Brain. 2015. https://doi.org/10.1186/s13041-015-0171-5.
5. Obis T, Besalduch N, Hurtado E, Nadal L, Santafe MM, Garcia N, et al. The novel protein kinase C epsilon isoform at the adult neuromuscular synapse: Location, regulation by synaptic activity-dependent muscle contraction through TrkB signaling and coupling to ACh release. Mol Brain. 2015. https://doi.org/10.1186/s13041-015-0098-x.
6. Santafé M, Lanuza MA, Garcia N, Tomàs J. Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. Eur J Neurosci. 2006;23:2048–56.
7. Simó A, Just-Borràs L, Cilleros-Mañé V, Hurtado E, Nadal L, Tomàs M, et al. Bdnf-TrKB signaling coupled to nPKCε and cPKCβI modulate the phosphorylation of the exocytotic protein MUNC18-1 during synaptic activity at the neuromuscular junction. Front Mol Neurosci. 2018. https://doi.org/10.3389/fnmol.2018.00207.
8. Simó A, Cilleros-Mañé V, Just-Borràs L, Hurtado E, Nadal L, Tomàs M, et al. nPKCε mediates SNAP-25 phosphorylation of Ser-187 in basal conditions and after synaptic activity at the neuromuscular junction. Mol Neurobiol. 2019;56:5346–64.
9. Cilleros-Mañé V, Just-Borràs L, Polishchuk A, Durán M, Tomàs M, Garcia N, et al. M1 and M2 mAChRs activate PDK1 and regulate PKC βI and ε and the exocytotic apparatus at the NMJ. FASEB J. 2021;35:e21724.
10. Haga T. Molecular properties of muscarinic acetylcholine receptors. Proc Jpn Acad Ser B Phys Biol Sci. 2013;89:226–56.
11. Garcia N, Santafé MM, Salon I, Lanuza MA, Tomàs J. Expression of muscarinic acetylcholine receptors (M1-, M2-, M3- and M4-type) in the neuromuscular junction of the newborn and adult rat. Histol Histopathol. 2005;20:733–43.
12. Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J. Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. Eur J Neurosci. 2003;17:119–27.
13. Santafé MM, Lanuza MA, Garcia N, Tomàs M, Tomàs J. Coupling of presynaptic muscarinic autoreceptors to serine kinases in low and high release conditions on the rat motor nerve terminal. Neuroscience. 2007;148:432–40.
14. Lanuza MA, Santafé MM, Garcia N, Besalduch N, Tomàs M, Obis T, et al. Protein kinase C isoforms at the neuromuscular junction: localization and specific roles in neurotransmission and development. J Anat. 2014;224:61–73.
15. Nagy G, Matti U, Nehring RB, Binz T, Rettig J, Neher E, et al. Protein kinase C-dependent phosphorylation of synaptosome-associated protein of 25 kDa at Ser187 potentiates vesicle recruitment. J Neurosci. 2002;22:9278–86.
16. Kraft AS, Anderson WB, Cooper HL, Sando JJ. Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. J Biol Chem. 1982;257:13193–6.
17. Südhof TC. Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. Neuron. 2013;80:675–90.
18. Sons MS, Verhage M, Plomp JJ. Role of Munc18-1 in synaptic plasticity at the myasthenic neuromuscular junction. Ann N Y Acad Sci. 2003;998:404–6.
19. Barclay JW, Craig TJ, Fisher RJ, Ciufo LF, Evans GJO, Morgan A, et al. Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. J Biol Chem. 2003;278:10538–45.
20. Morgan A, Craig TJ, Evans GJO. Use of phospho-specific antibodies to demonstrate phosphorylation of Munc18 / nSec1 in chromaffin cells. Cell Biology Chromaffin Cell. 2004;1:65–70.
21. Snyder DA, Kelly ML, Woodbury DJ. Snare complex regulation by phosphorylation. Cell Biochem Biophys. 2006;45:111–23.
22. Fujita Y, Sasaki T, Fukui K, Kotani H, Kimura T, Hata Y, et al. Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase c. J Biol Chem. 1996;271:7265–8.
23. Sørensen JB, Nagy G, Varoqueaux F, Nehring RB, Brose N, Wilson MC, et al. Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. Cell. 2003;114:75–86.

24. Leenders AGM, Sheng Z-H. Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. Pharmacol Ther. 2005;105:69–84.
25. Polishchuk A, Cilleros-Mañé V, Just-Borràs L, Balanyà-Segura M, Vandellòs Pont G, Silvera Simón C, et al. Synaptic retrograde regulation of the PKA-induced SNAP-25 and synapsin-1 phosphorylation. Cell Mol Biol Lett. 2023;28:17.
26. Hepp R, Cabaniols J-P, Roche PA. Differential phosphorylation of SNAP-25 *in vivo* by protein kinase C and protein kinase A. FEBS Lett. 2002;532:52–6.
27. Risinger C, Bennett MK. Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. J Neurochem. 1999;72:614–24.
28. Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, et al. Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. J Biol Chem. 1996;271:14548–53.
29. Yang Y, Craig TJ, Chen X, Ciufo LF, Takahashi M, Morgan A, et al. Phosphomimetic mutation of Ser-187 of SNAP-25 increases both syntaxin binding and highly Ca2+-sensitive exocytosis. J Gen Physiol. 2007;129:233–44.
30. Polishchuk A, Cilleros-Mañé V, Balanyà-Segura M, Just-Borràs L, Forniés-Mariné A, Silvera-Simón C, et al. BDNF/TrkB signalling, in cooperation with muscarinic signalling, retrogradely regulates PKA pathway to phosphorylate SNAP-25 and synapsin-1 at the neuromuscular junction. Cell Commun Signal. 2024. https://doi.org/10.1186/s12964-024-01735-2. (**22**).
31. Santafé MM, Garcia N, Lanuza MA, Tomàs M, Tomàs J. Interaction between protein kinase C and protein kinase A can modulate transmitter release at the rat neuromuscular synapse. J Neurosci Res. 2009;87:683–90.
32. Favreau P, Le Gall F, Benoit E, Molgó J. A review on conotoxins targeting ion channels and acetylcholine receptors of the vertebrate neuromuscular junction. Acta Physiol Pharmacol Ther Latinoam. 1999;49:257–67.
33. Cilleros-Mañé V, Just-Borràs L, Tomàs M, Garcia N, Tomàs JM, Lanuza MA. The M2 muscarinic receptor, in association to M1, regulates the neuromuscular PKA molecular dynamics. FASEB J. 2020;34(4):4934–55.
34. Watson N, Barnes PJ, Maclagan J. Actions of methoctramine, a muscarinic M2 receptor antagonist, on muscarinic and nicotinic cholinoceptors in guinea-pig airways in vivo and in vitro. Br J Pharmacol. 1992;105:107–12.
35. Caulfield MP. Muscarinic receptors—characterization, coupling and function. Pharmacol Ther. 1993;58:319–79.
36. Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, et al. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron. 2001;30:399–410.
37. Li X-M, Dong X-P, Luo S-W, Zhang B, Lee D-H, Ting AKL, et al. Retrograde regulation of motoneuron differentiation by muscle β-catenin. Nat Neurosci. 2008;11:262–8.
38. Wu H, Xiong WC, Mei L. To build a synapse: signaling pathways in neuromuscular junction assembly. Development. 2010;137:1017–33.
39. Martinez-Pena y Valenzuela I, Akaaboune M. The metabolic stability of the nicotinic acetylcholine receptor at the neuromuscular junction. Cells. 2021;10:358.
40. Martinez-Pena y Valenzuela I, Mouslim C, Akaaboune M. Calcium/calmodulin kinase II-dependent acetylcholine receptor cycling at the mammalian neuromuscular junction in vivo. J Neurosci. 2010;30:12455–65.
41. Brenner HR, Rudin W. On the effect of muscle activity on the end-plate membrane in denervated mouse muscle. J Physiol. 1989;410:501–12.
42. Schindelin J, Rueden CT, Hiner MC, Eliceiri KW. The imagej ecosystem: an open platform for biomedical image analysis. Mol Reprod Dev. 2015;82:518–29.
43. Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ. The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. J Neurosci Methods. 2008;172:250–4.
44. Hurtado E, Cilleros V, Just L, Simó A, Nadal L, Tomàs M, et al. Synaptic activity and muscle contraction increases PDK1 and PKCβ1 phosphorylation in the presynaptic membrane of the neuromuscular junction. Front Mol Neurosci. 2017. https://doi.org/10.3389/fnmol.2017.00270.
45. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9:676–82.
46. Dell RB, Holleran S, Ramakrishnan R. Sample size determination. ILAR J. 2002;43:207–13.
47. Ridgman WJ. Statistical Methods, 8th edn, by G. W. Snedecor & W. G. Cochran. Iowa State University Press (1989). J Agric Sci. 1990;115:153.
48. Tomàs J, Santafé MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, et al. Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. J Neurosci Res. 2014;92:543–54.
49. Wright MC, Potluri S, Wang X, Dentcheva E, Gautam D, Tessler A, et al. Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses. J Neurosci. 2009;29:14942–55.
50. Nadal L, Garcia N, Hurtado E, Simó A, Tomàs M, Lanuza MA, et al. Presynaptic muscarinic acetylcholine autoreceptors (M1, M2 and M4 subtypes), adenosine receptors (A1 and A2A) and tropomyosin-related kinase B receptor (TrkB) modulate the developmental synapse elimination process at the neuromuscular junction. Mol Brain. 2016. https://doi.org/10.1186/s13041-016-0248-9.
51. Malomouzh AI, Arkhipova SS, Nikolsky EE, Vyskočil F. Immunocytochemical demonstration of M(1) muscarinic acetylcholine receptors at the presynaptic and postsynaptic membranes of rat diaphragm endplates. Physiol Res. 2011;60:185–8.
52. Balanyà-Segura M, Polishchuk A, Just-Borràs L, Cilleros-Mañé V, Silvera C, Ardévol A, et al. Molecular adaptations of BDNF/NT-4 neurotrophic and muscarinic pathways in ageing neuromuscular synapses. Int J Mol Sci. 2024. https://doi.org/10.3390/ijms25158018.
53. Besalduch N, Tomàs M, Santafé MM, Garcia N, Tomàs J, Lanuza MA. Synaptic activity-related classical protein kinase C isoform localization in the adult rat neuromuscular synapse. J Comp Neurol. 2010;518:211–28.
54. Slutsky I, Parnas H, Parnas I. Presynaptic effects of muscarine on ACh release at the frog neuromuscular junction. J Physiol. 1999;514:769–82.
55. Georgiou J, Robitaille R, Charlton MP. Muscarinic control of cytoskeleton in perisynaptic glia. J Neurosci. 1999;19:3836–46.
56. Bélair E-L, Vallée J, Robitaille R. In vivo long-term synaptic plasticity of glial cells. J Physiol. 2010;588:1039–56.
57. Furlan I, Godinho RO. Developing skeletal muscle cells express functional muscarinic acetylcholine receptors coupled to different intracellular signaling systems. Br J Pharmacol. 2005;146:389–96.
58. Reyes R, Jaimovich E. Functional muscarinic receptors in cultured skeletal muscle. Arch Biochem Biophys. 1996;331:41–7.
59. Liu TP, Yu P-C, Liu I-M, Tzeng T-F, Cheng J-T. Activation of muscarinic M1 receptors by acetylcholine to increase glucose uptake into cultured C2C12 cells. Auton Neurosci. 2002;96:113–8.
60. Lanuza MA, Besalduch N, González C, Santafé MM, Garcia N, Tomàs M, et al. Decreased phosphorylation of delta and epsilon subunits of the acetylcholine receptor coincides with delayed postsynaptic maturation in PKC theta deficient mouse. Exp Neurol. 2010;225:183–95.
61. Li MX, Jia M, Yang LX, Jiang H, Lanuza MA, Gonzalez CM, et al. The role of the theta isoform of protein kinase C (PKC) in activity-dependent synapse elimination: evidence from the PKC theta knock-out mouse in vivo and in vitro. J Neurosci. 2004;24:3762–9.
62. Scherer NM, Nathanson NM. Differential regulation by agonist and phorbol ester of cloned m1 and m2 muscarinic acetylcholine receptors in mouse Y1 adrenal cells and in Y1 cells deficient in cAMP-dependent protein kinase. Biochemistry. 1990;29:8475–83.
63. Hao W, Xing-Jun W, Yong-Yao C, Liang Z, Yang L, Hong-Zhuan C. Up-regulation of M1 muscarinic receptors expressed in CHOm1 cells by panaxynol via cAMP pathway. Neurosci Lett. 2005;383:121–6.
64. Sterin-Borda L, Ganzinelli S, Berra A, Borda E. Novel insight into the mechanisms involved in the regulation of the m1 muscarinic receptor, iNOS and nNOS mRNA levels. Neuropharmacology. 2003;45:260–9.
65. Willets JM, Nelson CP, Nahorski SR, Challiss RAJ. The regulation of M1 muscarinic acetylcholine receptor desensitization by synaptic activity in cultured hippocampal neurons. J Neurochem. 2007;103:2268–80.
66. Fraser CM, Lee NH. Regulation of muscarinic receptor expression by changes in mRNA stability. Life Sci. 1995;56:899–906.
67. Newton AC. Regulation of protein kinase C. Curr Opin Cell Biol. 1997;9:161–7.
68. Ross EM, Berstein G. Regulation of the M1 muscarinic receptor-Gq-phospholipase C-beta pathway by nucleotide exchange and GTP hydrolysis. Life Sci. 1993;52:413–9.
69. Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA. Activation of protein kinase C triggers its ubiquitination and degradation. Mol Cell Biol. 1998;18:839–45.
70. Lee HW, Smith L, Pettit GR, Vinitsky A, Smith JB. Ubiquitination of protein kinase C-alpha and degradation by the proteasome. J Biol Chem. 1996;271:20973–6.
71. Kang B-S, French OG, Sando JJ, Hahn CS. Activation-dependent degradation of protein kinase Cη. Oncogene. 2000;19:4263–72.

72. Parker PJ, Bosca L, Dekker L, Goode NT, Hajibagheri N, Hansra G. Protein kinase C (PKC)-induced PKC degradation: a model for down-regulation. Biochem Soc Trans. 1995;23:153–5.
73. Olivier AR, Parker PJ. Identification of multiple PKC isoforms in Swiss 3T3 cells: differential down-regulation by phorbol ester. J Cell Physiol. 1992;152:240–4.
74. Caulfield MP. Muscarinic receptors - characterization, coupling and function. Pharmacol Ther. 1993;58:319–79.
75. Li M-X, Jia M, Jiang H, Dunlap V, Nelson PG. Opposing actions of protein kinase A and C mediate Hebbian synaptic plasticity. Nat Neurosci. 2001;4:871–2.
76. Garcia N, Balañà C, Lanuza MA, Tomàs M, Cilleros-Mañé V, Just-Borràs L, et al. Opposed actions of PKA isozymes (RI and RII) and PKC isoforms (cPKCβI and nPKCε) in neuromuscular developmental synapse elimination. Cells. 2019. https://doi.org/10.3390/cells8111304.
77. Wan M, Zhang W, Tian Y, Xu C, Xu T, Liu J, et al. Unraveling a molecular determinant for clathrin-independent internalization of the M2 muscarinic acetylcholine receptor. Sci Rep. 2015;5:11408.
78. Koenig JA, Edwardson JM. Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. Mol Pharmacol. 1996;49:351–9.
79. Nathanson NM. Synthesis, trafficking, and localization of muscarinic acetylcholine receptors. Pharmacol Ther. 2008;119:33–43.
80. Lambert L, Dubayle D, Fafouri A, Herzog E, Csaba Z, Dournaud P, et al. Endocytosis of activated muscarinic m2 receptor (m2R) in live mouse hippocampal neurons occurs via a clathrin-dependent pathway. Front Cell Neurosci. 2018. https://doi.org/10.3389/fncel.2018.00450.
81. Biddlecome GH, Berstein G, Ross EM. Regulation of phospholipase C-beta1 by Gq and m1 muscarinic cholinergic receptor. Steady-state balance of receptor-mediated activation and GTPase-activating protein-promoted deactivation. J Biol Chem. 1996;271:7999–8007.
82. Nathanson NM. A multiplicity of muscarinic mechanisms: enough signaling pathways to take your breath away. Proc Natl Acad Sci U S A. 2000;97:6245–7.
83. Strassheim D, Williams CL. P2y2 purinergic and M3 muscarinic acetylcholine receptors activate different phospholipase C-β isoforms that are uniquely susceptible to protein kinase C-dependent phosphorylation and inactivation. J Biol Chem. 2000;275:39767–72.
84. Liu M, Clarke CJ, Salama MF, Choi YJ, Obeid LM, Hannun YA. Co-ordinated activation of classical and novel PKC isoforms is required for PMA-induced mTORC1 activation. PLoS ONE. 2017;12:e0184818.
85. Moschella PC, Rao VU, McDermott PJ, Kuppuswamy D. Regulation of mTOR and S6K1 activation by the nPKC isoforms, PKCε and PKCδ, in adult cardiac muscle cells. J Mol Cell Cardiol. 2007;43:754–66.
86. Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev. 1998;50:279–90.
87. Jewell JL, Fu V, Hong AW, Yu F-X, Meng D, Melick CH, et al. GPCR signaling inhibits mTORC1 via PKA phosphorylation of Raptor. Elife. 2019. https://doi.org/10.7554/eLife.43038.
88. Melick CH, Lama-Sherpa TD, Curukovic A, Jewell JL. G-protein coupled receptor signaling and mammalian target of rapamycin complex 1 regulation. Mol Pharmacol. 2022;101:181–90.
89. Liu S, Shi D, Sun Z, He Y, Yang J, Wang G. M2-AChR mediates rapid antidepressant effects of scopolamine through activating the mTORC1-BDNF signaling pathway in the medial prefrontal cortex. Front Psychiatry. 2021. https://doi.org/10.3389/fpsyt.2021.601985.
90. Ham DJ, Börsch A, Lin S, Thürkauf M, Weihrauch M, Reinhard JR, et al. The neuromuscular junction is a focal point of mTORC1 signaling in sarcopenia. Nat Commun. 2020;11:4510.
91. Castets P, Ham DJ, Rüegg MA. The TOR pathway at the neuromuscular junction: more than a metabolic player? Front Mol Neurosci. 2020. https://doi.org/10.3389/fnmol.2020.00162.
92. Baraldo M, Geremia A, Pirazzini M, Nogara L, Solagna F, Türk C, et al. Skeletal muscle mTORC1 regulates neuromuscular junction stability. J Cachexia Sarcopenia Muscle. 2020;11:208–25.
93. Rion N, Castets P, Lin S, Enderle L, Reinhard JR, Eickhorst C, et al. mTOR controls embryonic and adult myogenesis via mTORC1. Development. 2019. https://doi.org/10.1242/dev.172460.
94. Bentzinger CF, Romanino K, Cloëtta D, Lin S, Mascarenhas JB, Oliveri F, et al. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. Cell Metab. 2008;8:411–24.
95. Matthews VB, Åström M-B, Chan MHS, Bruce CR, Krabbe KS, Prelovsek O, et al. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia. 2009;52:1409–18.
96. Mantilla CB, Zhan W-Z, Sieck GC. Neurotrophins improve neuromuscular transmission in the adult rat diaphragm. Muscle Nerve. 2004;29:381–6.
97. Mantilla CB, Stowe JM, Sieck DC, Ermilov LG, Greising SM, Zhang C, et al. TrkB kinase activity maintains synaptic function and structural integrity at adult neuromuscular junctions. J Appl Physiol. 2014;117:910–20.
98. Gómez-Pinilla F, Ying Z, Opazo P, Roy RR, Edgerton VR. Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. Eur J Neurosci. 2001;13:1078–84.
99. Gómez-Pinilla F, Ying Z, Roy RR, Molteni R, Edgerton VR. Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. J Neurophysiol. 2002;88:2187–95.
100. Hsiao Y-T, Shu W-C, Chen P-C, Yang H-J, Chen H-Y, Hsu S-P, et al. Presynaptic SNAP-25 regulates retinal waves and retinogeniculate projection via phosphorylation. Proc Natl Acad Sci U S A. 2019;116:3262–7.
101. Tomàs J, Lanuza MA, Santafé MM, Cilleros-Mañé V, Just-Borràs L, Balanyà-Segura M, et al. Muscarinic receptors in developmental axonal competition at the neuromuscular junction. Mol Neurobiol. 2022. https://doi.org/10.1007/s12035-022-03154-1.
102. Tomàs J, Garcia N, Lanuza MA, Santafé MM, Tomàs M, Nadal L, et al. Presynaptic membrane receptors modulate ACh release, axonal competition and synapse elimination during neuromuscular junction development. Front Mol Neurosci. Frontiers Media S.A.; 2017.

Publisher's Note