Molecular Neurobiology (2019) 56:5346–5364
https://doi.org/10.1007/s12035-018-1462-5

# nPKCε Mediates SNAP-25 Phosphorylation of Ser-187 in Basal Conditions and After Synaptic Activity at the Neuromuscular Junction

Anna Simó[1] · Victor Cilleros-Mañé[1] · Laia Just-Borràs[1] · Erica Hurtado[1] · Laura Nadal[1] · Marta Tomàs[1] · Neus Garcia[1] · Maria A. Lanuza[1] · Josep Tomàs[1]

Received: 5 September 2018 / Accepted: 18 December 2018 / Published online: 3 January 2019


## Abstract

Protein kinase C (PKC) and substrates like SNAP-25 regulate neurotransmission. At the neuromuscular junction (NMJ), PKC promotes neurotransmitter release during synaptic activity. Thirty minutes of muscle contraction enhances presynaptic PKC isoform levels, specifically cPKCβI and nPKCε, through retrograde BDNF/TrkB signaling. This establishes a larger pool of these PKC isoforms ready to promote neuromuscular transmission. The PKC phosphorylation site in SNAP-25 has been mapped to the serine 187 (Ser-187), which is known to enhance calcium-dependent neurotransmitter release in vitro. Here, we localize SNAP-25 at the NMJ and investigate whether cPKCβI and/or nPKCε regulate SNAP-25 phosphorylation. We also investigate whether nerve and muscle cell activities regulate differently SNAP-25 phosphorylation and the involvement of BDNF/TrkB signaling. Our results demonstrate that nPKCε isoform is essential to positively regulate SNAP-25 phosphorylation on Ser-187 and that muscle contraction prevents it. TrkB and cPKCβI do not regulate SNAP-25 protein level or its phosphorylation during neuromuscular activity. The results provide evidence that nerve terminals need both pre- and postsynaptic activities to modulate SNAP-25 phosphorylation and ensure an accurate neurotransmission process.

**Keywords** Neuromuscular junction · Muscle contraction · SNAP-25 · TrkB · PKC · Neurotransmission · Synaptic vesicles

**Abbreviations**

| | |
|---|---|
| 47/TrkB | Anti-TrkB antibody clone 47/TrkB |
| ACh | Acetylcholine |
| AChR | Acetylcholine receptor |
| BDNF | Brain-derived neurotrophic factor |
| $Ca^{2+}$ | Calcium ion |
| CNS | Central nervous system |
| cPKCβI | Conventional protein kinase C beta I |
| ECL | Enhanced chemiluminescence |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| HRP | Horseradish peroxidase |
| LAL | Levator auris longus |
| NMJ | Neuromuscular junction |
| nPKCε | Novel protein kinase C epsilon |
| NT-4/5 | Neurotrophin-4/5 |
| p75 | p75 neurotrophin receptor |
| PBS | Phosphate buffer saline |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PMA | Phorbol 12-myristate 13-acetate |
| pSNAP-25 | Phosphorylated synaptosomal-associated protein of 25 kDa |
| PVDF | Polyvinylidene difluoride |
| RACK | Receptor for activated C-kinase |
| SDS | Sodium dodecyl sulfate |
| Ser-187 | Serine 187 |
| SNAP-25 | Synaptosomal-associated protein of 25 kDa |
| SNARE | Soluble *N*-ethylmaleimide-sensitive factor attachment receptor proteins |
| TrkA | Tropomyosin receptor kinase A |
| TrkB | Tropomyosin receptor kinase B |

Anna Simó, Victor Cilleros-Mañé and Laia Just-Borràs contributed equally to this work and Neus Garcia, Maria A. Lanuza and Josep Tomàs contributed equally to this work.

✉ Maria A. Lanuza
mariaangel.lanuza@urv.cat

✉ Josep Tomàs
josepmaria.tomas@urv.cat

[1] Unitat d'Histologia i Neurobiologia (UHNEUROB), Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Spain

| | |
|---|---|
| TrkC | Tropomyosin receptor kinase C |
| α-BTX | α-Bungarotoxin |
| $\beta IV_{5-3}$ | cPKCβI-specific translocation inhibitor peptide |
| $\varepsilon V_{1-2}$ | nPKCε-specific translocation inhibitor peptide |
| μ-CgTx-GIIIB | μ-Conotoxin GIIIB |
| VGCC | Voltage-gated calcium channels |

## Introduction

At the neuromuscular junction (NMJ), protein kinase C (PKC) signaling regulates neurotransmitter release and neuromuscular function [1–7]. PKC phosphorylates several proteins involved in exocytosis including SNAP-25 (synaptosomal-associated protein of 25 kDa) [8–10]. SNAP-25, 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 [11, 12].

SNAP-25 is phosphorylated by PKC at serine 187 (Ser-187), a critical residue for calcium-triggered exocytosis [8–10, 13–15]. This phosphorylation occurs in response to synaptic activity and elevated intracellular calcium levels and is involved in vesicle pool refilling [15–17]. Therefore, the absence of SNAP-25 causes a complete block of fast calcium-triggered exocytosis, as the primed pool of synaptic vesicles becomes empty [18]. Accordingly, changes in the phosphorylation residue decrease vesicle secretion [19] and the overexpression of a phospho-Ser-187 mimicking mutant accelerates vesicle recruitment after the emptying of the releasable vesicle pool [16]. Therefore, PKC phosphorylation of SNAP-25 is required for fast vesicle pool refilling in response to synaptic activity or elevated intracellular calcium [16, 17]. Moreover, SNAP-25 contributes to the coupling between exocytosis and endocytosis by participating in slow, clathrin-dependent endocytosis at hippocampal synapses [20]. Also, pSNAP-25 negatively regulates the voltage-gated calcium channels (VGCC) [21] and it has been shown that it is negatively implicated in the control of neuronal calcium responsiveness to depolarization [22].

Although SNAP-25 is a PKC target, there is no information about which PKC isoform regulates its phosphorylation. The cPKCβI and nPKCε isoforms are good candidates because they are exclusively presynaptic at the NMJ, synaptic activity modulates them, and, in turn, they regulate acetylcholine (ACh) release [23–26]. Thus, in this study, we localized SNAP-25 at the NMJ and investigated whether cPKCβI and/or nPKCε regulate its phosphorylation. Because nerve and muscle cell activities act differently on these PKC isoforms through BDNF/TrkB signaling [23–26], we also tested the effect of nerve and muscle cell activities on SNAP-25 phosphorylation and the involvement of BDNF/TrkB signaling. The main result shows that nPKCε promotes SNAP-25 phosphorylation on Ser-187 during synaptic activity, muscle contraction prevents it, and neither TrkB nor cPKCβI is involved during neuromuscular activity.

## Material and Methods

### Animals

Young adult Sprague-Dawley rats (30–40 days; Criffa, Barcelona, Spain; RRID:RGD_5508397) were cared for in accordance with the guidelines of the European Community Council Directive for the humane treatment of laboratory animals. All the procedures were approved by the Animal Experimentation Ethics Committee of the Universitat Rovira i Virgili.

### Antibodies

Primary antibodies were purchased from Santa Cruz Biotechnology: polyclonal rabbit anti-PKCε (Cat# sc-214 RRID:AB_2237729), rabbit anti-pPKCε (Ser 729) (Cat# sc-12355 RRID: AB_654732), and monoclonal mouse anti-GAPDH (Cat# sc-32233 RRID: AB_627679); from Developmental Studies Hybridoma Bank: monoclonal mouse anti-$Na^+/K^+$-ATPase (Cat# a6F RRID: AB_528092); from ThermoFisher-Invitrogen: polyclonal rabbit anti-pSNAP-25 (Ser-187) (Cat# PA5-35406 RRID: AB_2552716); from CST: monoclonal rabbit anti-SNAP-25 (D9A12) (Cat# 5309 RRID: AB_10624866). The anti-PKCε and anti-SNAP-25 antibodies recognized both phosphorylated and non-phosphorylated forms.

Immunohistochemistry was performed with antibodies widely used as markers of the NMJ, which were the monoclonal mouse anti-syntaxin (Cat# S0664 RRID: AB_477483) from Sigma and the monoclonal mouse anti-S-100 (Cat# AM10036FC-N RRID: AB_1622661) from Acris. Postsynaptic acetylcholine receptors (AChRs) were detected with α-bungarotoxin (α-BTX) conjugated to TRITC from Molecular Probes (Cat# T1175).

Secondary antibodies conjugated to HRP were donkey anti-rabbit from Jackson Immunoresearch Labs (Cat# 711-035-152 RRID: AB_10015282) and rabbit anti-mouse from Sigma (Cat# A9044 RRID: AB_258431) for Western blot. For immunohistochemistry, we used donkey anti-rabbit or anti-mouse conjugated to Alexa Fluor 488 and Alexa Fluor 647 from Molecular Probes (Cat# A21206 RRID: AB_141708; Cat# A21202 RRID: AB_141607; Cat# A-31573 RRID: AB_2536183; Cat# A-31571 RRID: AB_2536181).

We omitted primary antibodies in some immunohistochemical and Western blot procedures as negative controls. They never exhibited positive staining or HRP activity with the respective procedures. In double-staining protocols, the

omission of either one of the two primary antibodies abolished the corresponding staining without cross-reaction with the other primary antibody. Antibody specificity against PKC isoforms is shown in [24, 26]. 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 [27–29], making cross-reactivity less likely. On the other hand, phosphorylated SNAP-25 (pSNAP-25) at Ser-187, which is the phosphorylation site for PKC in vitro [9, 10], was detected by an antibody raised against the sequence (C)MEKADpSNKTRI, corresponding to the residues 182–192 of the protein. This sequence differs from SNAP-23 (identity percentages in rat: 73%); SNAP-47 (9%); and SNAP-29 (27%). Sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw) [30]. Moreover, the phosphospecificity was proven by the absence of signal after incubation with the antigen phosphopeptide [31] and after treatment with lambda phosphatase (manufacturer's datasheet).

## Reagents

PKC activation was induced by applying high calcium ($Ca^{2+}$) concentration (5 mM) or phorbol 12-myristate 13-acetate (PMA) (10 nM in muscle [3]; 100 nM in brain [32]) during 30 min. PKC inhibition was induced applying low $Ca^{2+}$ concentration (0.25 mM) or specific translocation inhibitors. The role of calcium was further investigated with BAPTA-AM following the protocol by Losavio and Muchnik [33]: $3 \times 10^{-7}$ mol BAPTA-AM/30 mg muscle in a $Ca^{2+}$-free buffer for 2 h. The specific cPKCβI inhibitor $\beta IV_{5\text{-}3}$ peptide [34, 35] was kindly provided by Dr. Mochly-Rosen from Stanford University and the specific nPKCε inhibitor $\varepsilon V_{1\text{-}2}$ peptide [36] from MERCK. The intracellular $\beta IV_{5\text{-}3}$ peptide (CKLFIMN) and $\varepsilon V_{1\text{-}2}$ peptide (EAVSLKPT), both < 40 amino acids, were designed and their specificity tested by the Mochly-Rosen Lab [36, 37]. The application of these peptides did not affect similar isoforms. Thus, blocking cPKCβI did not affect cPKCβII [38] and neither blocking nPKCε affected nPKCδ [36]. In addition, the classic calcium-dependent cPKCs are not inhibited with the peptide $\varepsilon V_{1\text{-}2}$ [39, 40]. Furthermore, there is evidence that shows that the effects found using the nPKCε-specific translocation inhibitor peptide can be confirmed when nPKCε knockout mice are used [41, 42]. Indeed, multiple sequence alignment reveals that $\beta IV_{5\text{-}3}$ peptide shares 100% identity with cPKCβI (Uniprot ID: P68403-1) and 0% identity with nPKCε (Uniprot ID: P09216). Additionally, $\varepsilon V_{1\text{-}2}$ peptide shares 0% identity with cPKCβI and 100% identity with nPKCε. Moreover, we tested that at the NMJ, $\varepsilon V_{1\text{-}2}$ is able to significantly decrease nPKCε and phospho-nPKCε in the membrane [26, 43] and that $\beta IV_{5\text{-}3}$ decreases cPKCβI and phospho-cPKCβI [24, 43]. Also, $\varepsilon V_{1\text{-}2}$ and $\beta IV_{5\text{-}3}$ affect in opposite way phosphorylation of Munc18-1 at the NMJ [43]. Dimethyl sulfoxide was used as vehicle. Once inside nerve terminals—the only NMJ component expressing cPKCβI and nPKCε—these peptides disrupt the interaction between the receptor for activated C-kinase (RACK) and the respective isoform. Working concentrations were optimized to 10 μM for the $\beta IV_{5\text{-}3}$ peptide [24] and 100 μM for the $\varepsilon V_{1\text{-}2}$ peptide [26]. The difference in concentration was due to $\beta IV_{5\text{-}3}$ peptide being connected to a deliverer peptide to enhance cell penetration.

TrkB inhibition was performed using an anti-TrkB antibody (clone 47/TrkB) from BD Transduction Laboratories (Cat# 610101 RRID: AB_397507). This antibody has been functionally validated as a TrkB selective inhibitor, reducing BDNF effects without binding to TrkA, TrkC, or $p75^{NTR}$ [44]. The working solution was 10 μg/ml. For exogenous BDNF incubations, we used h-BDNF (Alomone Labs; Cat# B-250) in a working solution of 10 mM.

Muscle contraction was blocked with μ-conotoxin GIIIB (μ-CgTx-GIIIB, Alomone Labs Ltd) for the presynaptic stimulation treatment. This peptide selectively inhibits sarcolemmal voltage-dependent sodium channels without altering ACh signaling [45]. It was supplied as lyophilized powder of > 99% purity and the working concentration was 1.5 μM [46].

## Presynaptic Electrical Stimulation of Muscles

Diaphragm muscles were dissected conserving the phrenic nerve as previously described [23, 26]. From each animal, one hemidiaphragm underwent the experimental condition while the other was the control. Hemidiaphragms were in oxygenated (95/5% of $O_2/CO_2$) Ringer's solution (mM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, $NaH_2PO_4$ 1, $NaHCO_3$ 12, and glucose 12.1) at room temperature. Muscles were stimulated ex vivo through the phrenic nerve at 1 Hz, which allows the maintenance of different tonic functions without depleting synaptic vesicles, during 30 min unless otherwise noted, using the A-M Systems 2100 isolated pulse generator (A-M System). Visible contractions served to verify successful nerve dissection and stimulation. Three main experiments were performed to distinguish the effects of synaptic activity from those of muscle activity. To assess synaptic activity, we compared presynaptically stimulated muscles whose contraction was blocked by μ-CgTx-GIIIB with non-stimulated muscles also incubated with μ-CgTx-GIIIB to control for nonspecific effects of the blocker. To assess the effect of muscle contraction per se, we compared stimulated/contracting muscles with stimulated/non-contracting muscles. To assess the complete effect of synaptic activity with the resulting muscle contraction, we compared stimulated/contracting muscles

with non-stimulated muscles, without exposure to μ-CgTx-GIIIB (for further information, see [24]).

### Western Blot

Western blot was performed as described in [43]. In brief, diaphragm muscle, brain regions, and spinal cord were quickly removed and transferred to ice-cold dissection Ringer. After treatment, muscles were homogenized in ice-cold lysis buffer—the membrane/cytosol fractioning required detergent-free buffer—with an overhead stirrer (VWR International) and protein lysates were collected and centrifuged. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and $Na^+/K^+$-ATPase immunoreactivity were used to determine the purity of the membrane/cytosol fractionation.

Protein concentration was determined using the DC protein assay (Bio-Rad) and, then, samples of 30 μg were separated by SDS-polyacrylamide electrophoresis and electrotransferred to PVDF membranes (Hybond™-P; Amersham, GE Healthcare). Membranes were blocked and incubated with a primary antibody overnight at 4 °C and the corresponding secondary antibody for 1 h.

Blots were visualized with the ChemiDoc XRS+ Imaging System (Bio-Rad) and the ECL kit from Amersham Life Science. Sample loading and antibodies were optimized to guarantee the linear range during the first minute of exposure. The densitometry of the bands was obtained with the MetaMorph Microscopy Automation and Image Analysis Software. The integrated optical density of the bands was normalized in relation to (1) background values and (2) total protein staining (Sypro Ruby, Bio-Rad). The relative variations between the experimental and control samples were calculated from the same image. Data are mean values ± SEM. Graphic artwork was created with Excel. Statistical significance of the differences between groups was evaluated under the Wilcoxon test or the Student *t* test and the normality of the distributions was evaluated with the Kolmogorov-Smirnov test. The criterion for statistical significance was $p < 0.05$ versus the control.

### Immunohistochemistry and Confocal Microscopy

Whole muscles were processed by immunohistochemistry to detect and localize SNAP-25 at the NMJ. Diaphragm and *levator auris longus* (LAL) muscles from young adult rats were fixed with 4% paraformaldehyde for 30 min. After fixation, the muscles were rinsed with phosphate buffer saline (PBS) and incubated in 0.1 M glycine in PBS. The muscles were permeabilized with 0.5% Triton X-100 in PBS, and non-specific binding was blocked with 4% bovine serum albumin. Then, they were incubated overnight at 4 °C in mixtures of primary antibodies. Finally, the muscles were incubated for 4 h at room temperature in a mixture of appropriate secondary antibodies and α-BTX. At least three muscles were used for the negative controls as described previously [23]. Localization of SNAP-25 at the NMJ was observed with a laser scanning confocal microscope (Nikon TE2000-E). We took special consideration to avoid channel contamination. 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. At least 6 muscles and 25 endplates per muscle were studied.

## Results

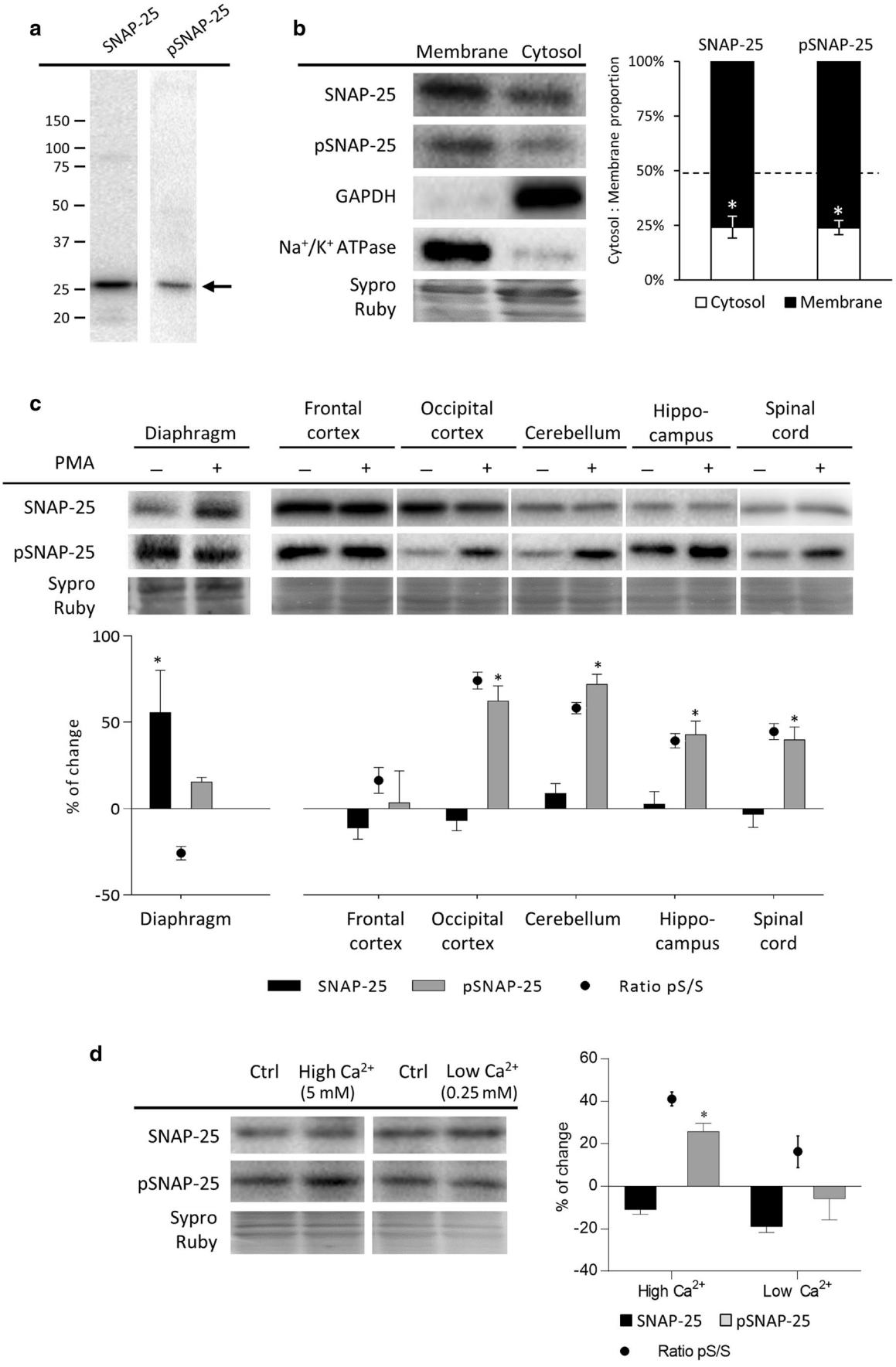
### SNAP-25 and pSNAP-25 in the Adult Skeletal Muscle

SNAP-25 and pSNAP-25 presence was determined in the diaphragm, brain regions, and spinal cord by Western blot. In all tissues, these antibodies only recognized the corresponding protein, labeling a 28-kDa band for both, which corresponded to the manufacturers' predicted molecular weight (Fig. 1a). The Western blot experiments revealed smaller amounts of SNAP-25 and pSNAP-25 in the diaphragm muscle compared to the central nervous tissues.

Figure 1b shows that both SNAP-25 and pSNAP-25 are predominant in the membrane but are also present in the cytosol. The cytosolic marker GAPDH was confined in the cytosol fraction and only slightly detectable in the membrane fraction whereas the membrane protein $Na^+/K^+$-ATPase was predominant in the membrane fraction and only slightly detectable in the cytosol fraction, confirming the successful protein extraction.

Because Ser-187 phosphorylation of SNAP-25 occurs in response to PKC stimulus in vitro [9, 16, 47], we investigated whether this activation also occurs in isolated muscles. Figure 1c shows that SNAP-25 increased (72.55% ± 7.55; $p < 0.05$) after the treatment with the PKC pan-activator PMA (10 nM, 30 min) while it did not affect the phosphorylation in the diaphragm, thus decreasing the ratio pSNAP-25/SNAP-25 (− 33.18% ± 5.56, $p < 0.05$). This was in contrast to the effect that PMA had in the CNS regions we studied—

**Fig. 1** SNAP-25 and pSNAP-25 characterization in the adult skeletal muscle and central nervous system. **a** Western blot bands from diaphragm showing the specificity of the antibodies SNAP-25 and pSNAP-25 (Ser-187). **b** Western blot bands and quantification of membrane and cytosol distribution of SNAP-25 and pSNAP-25. Both are mainly located in the membrane. GAPDH is exclusive of the cytosol and $Na^+/K^+$-ATPase, of the membrane. **c** Western blot bands and quantification of SNAP-25 and pSNAP-25 after PMA treatment in diaphragm, brain, and spinal cord. **d** Western blot bands and quantification of SNAP-25 and pSNAP-25 after different calcium concentrations. High $Ca^{2+}$ concentration increases SNAP-25 phosphorylation ratio levels while low $Ca^{2+}$ does not affect it. Data are mean percentage ± SEM, $^*p < 0.05$ ($n = 5$)


a
SNAP-25
pSNAP-25
150
100
75
50
37
25
20
b
Membrane
Cytosol
SNAP-25
pSNAP-25
GAPDH
Na+/K+ ATPase
Sypro Ruby
SNAP-25
pSNAP-25
100%
75%
50%
25%
0%
Cytosol : Membrane proportion
Cytosol
Membrane
c
Diaphragm
Frontal cortex
Occipital cortex
Cerebellum
Hippo-campus
Spinal cord
PMA
–
+
SNAP-25
pSNAP-25
Sypro Ruby
100
50
0
-50
% of change
Diaphragm
Frontal cortex
Occipital cortex
Cerebellum
Hippo-campus
Spinal cord
SNAP-25
pSNAP-25
Ratio pS/S
d
Ctrl
High Ca2+ (5 mM)
Ctrl
Low Ca2+ (0.25 mM)
SNAP-25
pSNAP-25
Sypro Ruby
60
40
20
0
-20
-40
% of change
High Ca2+
Low Ca2+
SNAP-25
pSNAP-25
Ratio pS/S

except the frontal cortex—and in neuron and chromaffin cell cultures [9, 21]. Figure 1c shows that PMA increases pSNAP-25 levels in occipital cortex, cerebellum, hippocampus, and spinal cord—which are the regions with lower basal pSNAP-25 levels—without affecting SNAP-25 levels.

Intracellular $Ca^{2+}$, which triggers exocytosis, has also been shown to induce the phosphorylation of SNAP-25 on Ser-187 in chromaffin cells [9, 14, 21]. In order to verify whether $Ca^{2+}$ also promotes this phosphorylation at the NMJ, we treated muscles with high (5 mM) or low (0.25 mM) $Ca^{2+}$ for 30 min. Figure 1d shows that high $Ca^{2+}$ enhanced pSNAP-25 (25.65% ± 3.88; $p < 0.05$), without changing the protein levels. Accordingly, the ratio pSNAP-25/SNAP-25 increased (41.08% ± 3.24, $p < 0.05$). On the contrary, incubation in low $Ca^{2+}$ did not modify SNAP-25 or pSNAP-25. Furthermore, $Ca^{2+}$ chelation with BAPTA-AM reduced both SNAP-25 (− 40.88% ± 8.26, $p < 0.05$) and pSNAP-25 (− 31.35% ± 7.92, $p < 0.05$) without affecting the ratio pSNAP-25/SNAP-25 (16.11% ± 5.24, $p > 0.05$).

## SNAP-25 Is Located in the Nerve Terminal at the Neuromuscular Junction

We used immunofluorescence staining coupled with confocal microscopy analysis to determine the location of SNAP-25 at the adult NMJ. Experiments were performed in the diaphragm and LAL muscles, being SNAP-25 immunoreactivity identical in both. All pictures in Fig. 2a show intense immunoreactivity for SNAP-25 in the synaptic area, identified with AChR labeling. Figure 2a (a, b) shows a NMJ with double labeling: AChRs in red and SNAP-25 in green. It can also be observed that SNAP-25 labeling is strongly concentrated over the AChR areas. Figure 2a (c–f) shows NMJs with triple labeling: SNAP-25 in green, AChRs in red, and nerve terminals (syntaxin; Fig. 2a (c, d)) or Schwann cells (S-100; Fig. 2a (e, f)) in blue. Images in (b), (d), and (f) are confocal optical sections of the images (a), (c), and (e) respectively. The *en side* image in Fig. 2a (d) shows detailed SNAP-25 green immunolabeling at the presynaptic position concentrated over the red postsynaptic gutters and colocalized with syntaxin. Figure 2a (e, f) shows that there is no colocalization between SNAP-25 and S-100 from the Schwann cell. The muscle cell has not been labeled. Thus, SNAP-25 is located in the nerve terminal at the neuromuscular junction.

Images in Fig. 2b allow to appreciate subcellular protein distribution at the NMJ. Images show NMJs with triple labeling: SNAP-25 in green, AChRs in red, and nerve terminals (syntaxin) in blue. Higher magnification images (d, e, and f) are confocal optical sections of the images (a), (b), and (c) respectively. It is observed that SNAP-25 is concentrated at the nerve terminals with higher immunosignal in specific areas (arrows), which may correspond to places close to the active zones.

**Fig. 2** SNAP-25 is exclusively located in the presynaptic component of the NMJ. **a** Multiple immunofluorescence-stained LAL muscles visualized at the confocal microscope. (a, b) NMJ with double labeling: AChRs in red and SNAP-25 in green. SNAP-25 labeling is strongly concentrated over the AChR areas ((b) is a confocal optical section of (a)). (c–f) show NMJs with triple labeling: AChRs in red, nerve terminals (syntaxin; (c, d)) or Schwann cells (S-100; (e, f)) in blue and SNAP-25 in green. Images in (b), (d), and (f) are confocal optical sections of the images (a), (c), and (e) respectively. The en side image in (d) shows detailed SNAP-25 green immunolabeling at the presynaptic position concentrated over the red postsynaptic gutters and colocalized with syntaxin. (e, f) show that there is no colocalization between SNAP-25 and S-100 from the Schwann cell. Scale bars = 10 μm. **b** NMJs with triple labeling: SNAP-25 in green, AChRs in red, and nerve terminals (syntaxin) in blue. Higher magnification images (d, d', e, e', and f, f') are confocal optical sections of the images (a), (b), and (c) respectively. SNAP-25 is concentrated at the nerve terminals with higher immunosignal in concrete areas (arrows), which may correspond to places close to the active zones. Scale bars (a, b, and c) = 10 μm; scale bars (d, d', e, e', and f, f') = 2.5 μm

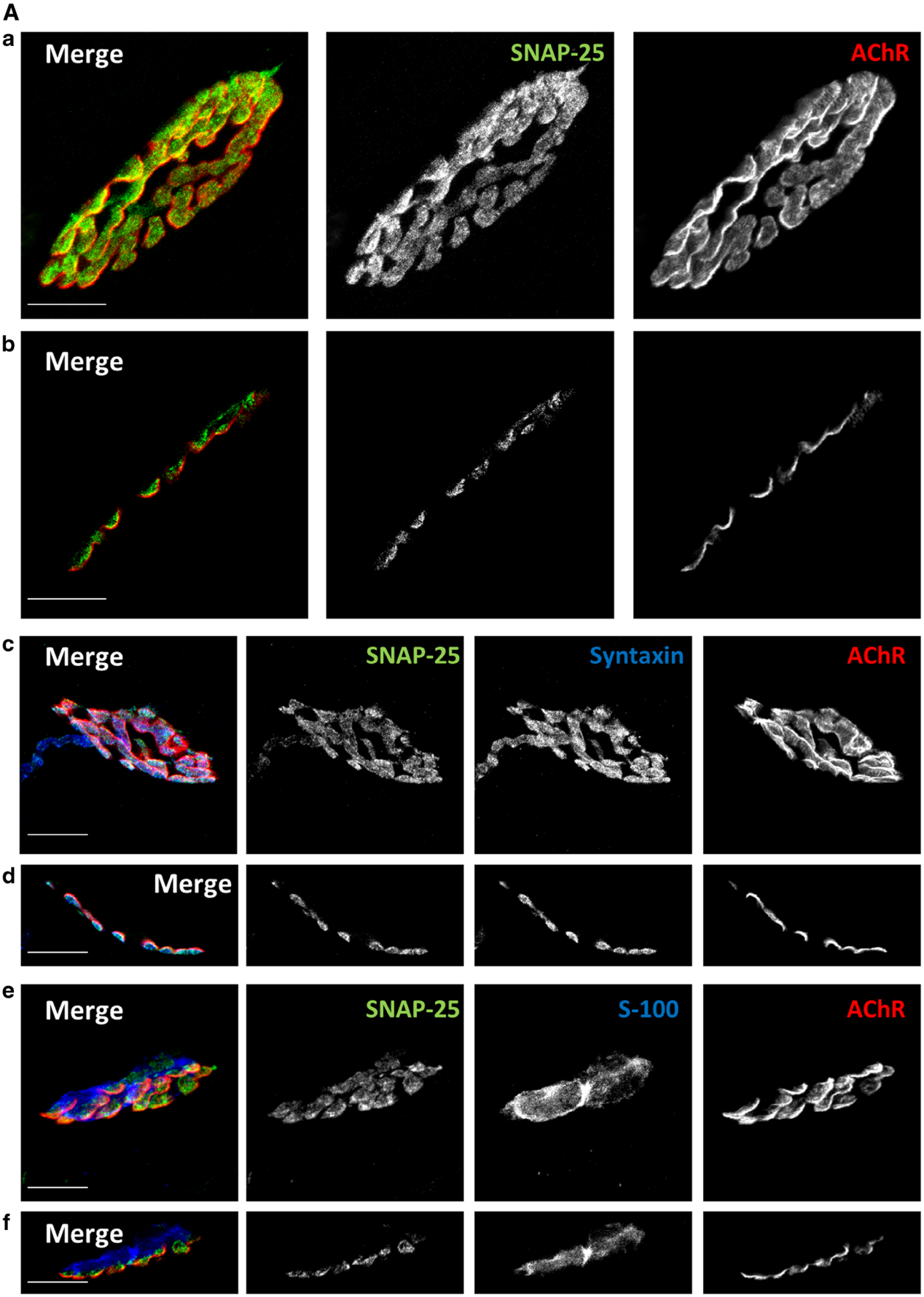
## Synaptic Activity Increases SNAP-25 Phosphorylation at the NMJ

Next, we analyzed whether synaptic activity regulates SNAP-25 protein levels. We separated the effect of the presynaptic stimulation (and synaptic transmission) from the effect of the muscle cell contraction, by performing experiments in which contraction was inhibited [24]. As previously described, muscle contraction was inhibited using μ-CgTx-GIIIB [45], which preserves neurotransmission. Figure 3a shows that nerve stimulation (1 Hz for 30 min) increased pSNAP-25 levels (46.12% ± 6.31; $p < 0.05$), without changing SNAP-25 (Fig. 3a). In concordance, the ratio pSNAP-25/SNAP-25 increased (51.99% ± 9.91; $p < 0.05$). Moreover, Fig. 3b shows that under electrical stimulation through the nerve, pSNAP-25 increased in the membrane fraction (20.92% ± 4.32, $p < 0.05$), indicating that the increased pSNAP-25 after stimulation is associated with the membrane.

We analyzed pSNAP-25 and SNAP-25 along the time of stimulation (1, 10, and 30 min, 1 Hz) (Fig. 3c). Stimulation for 1 and 10 min did not modify SNAP-25 or pSNAP-25 levels and only longer stimulations (30 min) enhanced pSNAP-25 levels.

## nPKCε Regulates Synaptic-Induced SNAP-25 Phosphorylation at the NMJ

Next, we tested which PKC isoforms are involved in SNAP-25 phosphorylation during basal conditions and after presynaptic stimulation. cPKCβI and nPKCε isoforms were chosen due to their exclusive presynaptic location at the NMJ [23] and their ability to regulate ACh release [24, 25]. Their activity was blocked with the specific translocation inhibitor peptides betaI $V_{5-3}$ ($\beta IV_{5-3}$) or epsilon $V_{1-2}$ ($\varepsilon V_{1-2}$), respectively [34, 36, 48]. Specificity of the inhibitor peptides has been previously tested [24, 26, 36, 38–43] and it is described in the "Material and Methods" section.


A
a
Merge
SNAP-25
AChR
b
Merge
c
Merge
SNAP-25
Syntaxin
AChR
d
Merge
e
Merge
SNAP-25
S-100
AChR
f
Merge

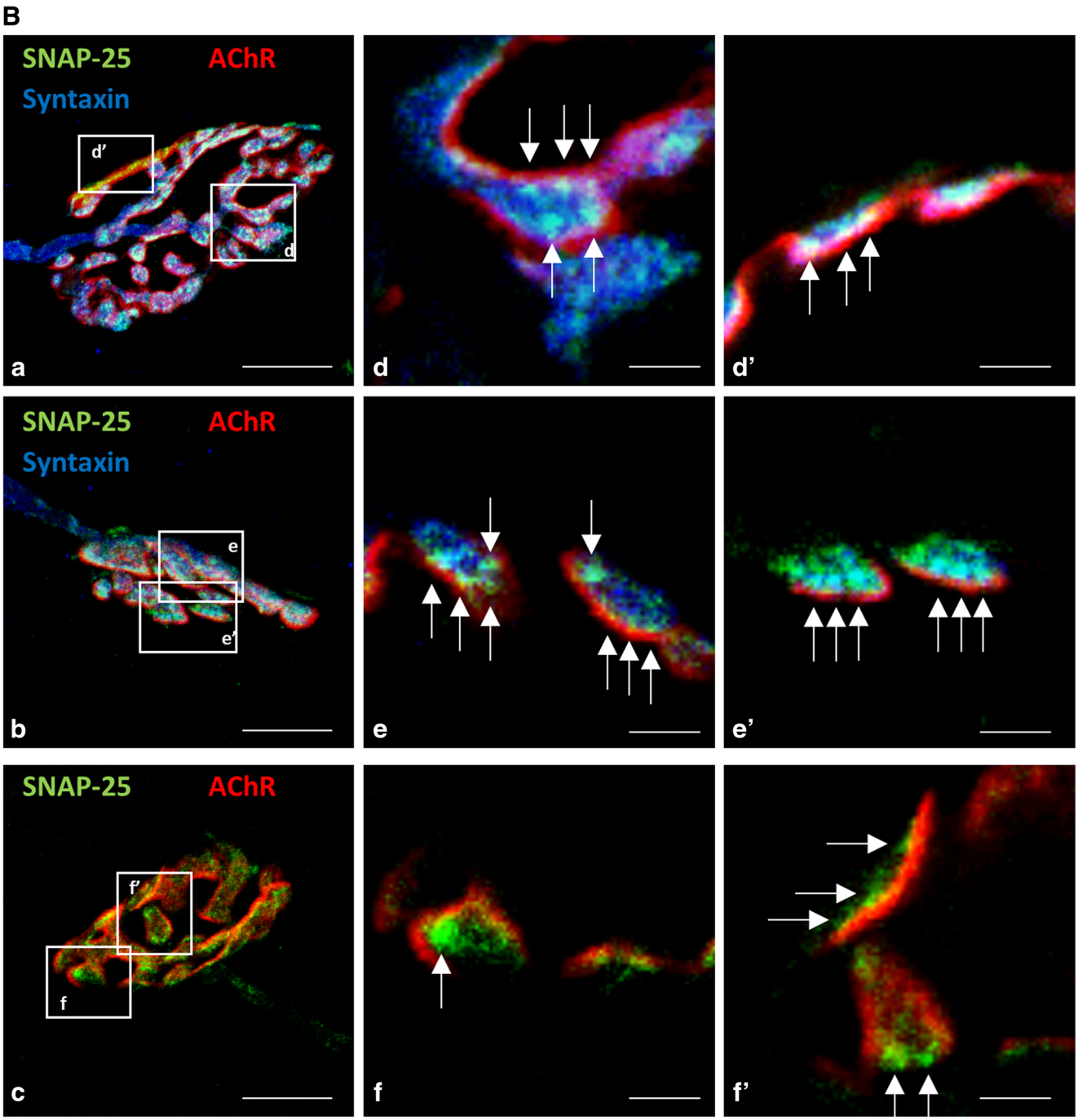



**Fig. 2** (continued)

## nPKCε

Figure 4a shows that the peptide $\varepsilon V_{1\text{-}2}$ incubated during 30 min in basal conditions decreases SNAP-25 and pSNAP-25. To link the effects of synaptic activity with nPKCε, we stimulated muscles (1 Hz, 30 min) after a previous incubation with $\varepsilon V_{1\text{-}2}$ (100 μM, 30 min). We found a significant decrease in pSNAP-25 level ($-27.53\% \pm 7.23$; $p < 0.05$) without changes in SNAP-25 ($-8.37\% \pm 5.22$; $p > 0.05$) (Fig. 4a). In concordance, the ratio pSNAP-25/SNAP-25 significantly decreased ($-20.91\% \pm 4.96$; $p < 0.05$), indicating that nPKCε enhances phosphorylation of SNAP-25 in synaptic activity.

Furthermore, to know whether nPKCε is responsible of the increase of pSNAP-25 induced by the nerve stimulation, we studied the effect of synaptic activity after $\varepsilon V_{1\text{-}2}$ inhibition (Fig. 4a). We found no change on SNAP-25 ($6.4\% \pm 3.11$; $p > 0.05$) nor pSNAP-25 levels ($2.98\% \pm 1.81$; $p > 0.05$) in comparison with non-stimulated, $\varepsilon V_{1\text{-}2}$

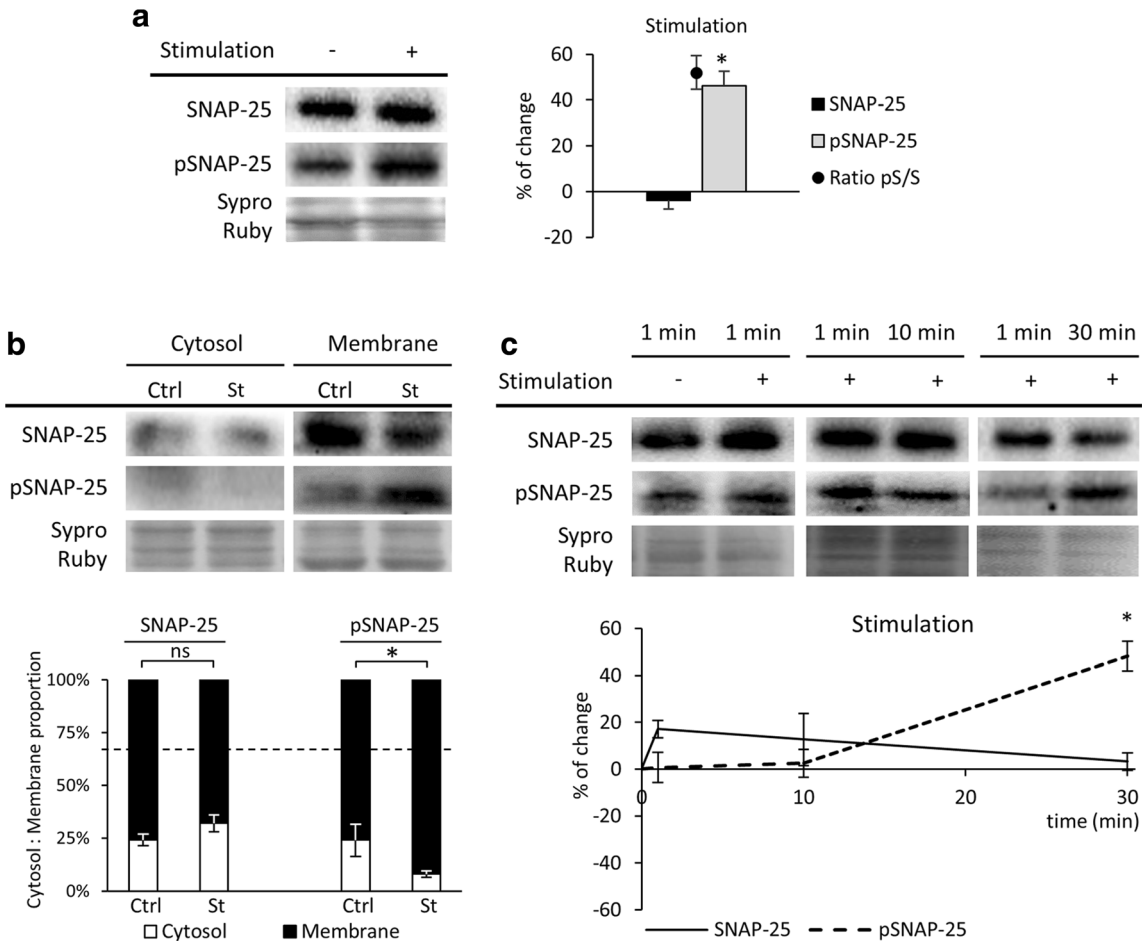



**Fig. 3** Synaptic activity increases SNAP-25 phosphorylation at the NMJ. **a** Western blot bands and quantification of diaphragm muscles show that pSNAP-25 increases after stimulation without contraction. **b** Western blot bands and quantification show that pSNAP-25 increases in the membrane fraction while SNAP-25 does not change. **c** Western blot bands and their representation show SNAP-25 and pSNAP-25 modulation through different stimulation times without contraction. While pSNAP-25 progressively increases from 10 to 30 min, SNAP-25 is sustained until 30 min. Data are mean percentage ± SEM, $*p < 0.05$ ($n = 5$)

inhibited muscles. Therefore, nPKCε is completely responsible of the SNAP-25 phosphorylation induced by synaptic activity as $\varepsilon V_{1\text{-}2}$ prevents it.

Figure 4b shows that, in basal conditions, nPKCε is present mainly in the membrane and pnPKCε is almost equally distributed. It was previously known that electrical stimulation significantly decreases nPKCε and pnPKCε levels [26], and now we show that this decrease occurs mainly in the membrane. This could be explained by membrane pnPKCε being degraded after its activity under stimulation [49–53], further relating its action on SNAP-25.

## cPKCβI

We also analyzed the effect of blocking cPKCβI function on SNAP-25 phosphorylation in the skeletal muscle. Figure 5 shows that the peptide $\beta IV_{5\text{-}3}$ did not change SNAP-25 or pSNAP-25 neither in basal conditions (ratio pSNAP-25/SNAP-25: $-13.82\% \pm 5.31$; $p > 0.05$) nor synaptic activity (ratio pSNAP-25/SNAP-25: $-11.19\% \pm 6.1$; $p > 0.05$). This indicates that, among the two PKC isoforms studied, cPKCβI does not contribute to SNAP-25 phosphorylation and only nPKCε regulates the phosphorylation of SNAP-25 during synaptic activity at the NMJ.

## BDNF/TrkB Pathway Does Not Regulate pSNAP-25 Under Stimulation at the NMJ

Presynaptic activity enhances BDNF/TrkB signaling pathway to regulate cPKCβI and nPKCε levels [24, 26, 43]. To study whether the activation of TrkB affects pSNAP-25 levels, we selectively suppressed TrkB activity in basal conditions and in nerve-stimulated diaphragm muscles. We used an anti-TrkB antibody (47/TrkB) which effectively inhibits BDNF binding to TrkB receptor [54]. Figure 6a shows that exogenous BDNF in basal conditions reduces SNAP-25 ($-27.86\% \pm 4.66$; $p < 0.05$) and pSNAP-25 ($-32.91\% \pm 9.77$; $p < 0.05$) without affecting the ratio ($-7.00\% \pm 4.40$; $p > 0.05$). Accordingly, the inhibitor

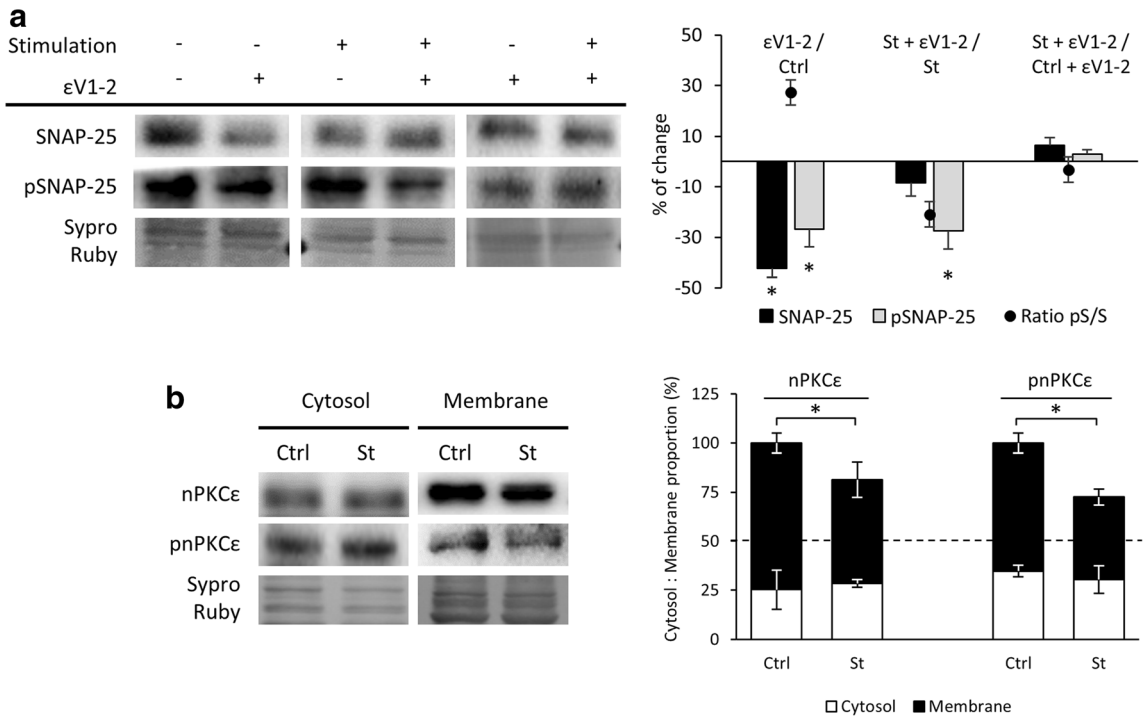



**Fig. 4** nPKCε regulates synaptic activity-induced SNAP-25 phosphorylation. **a** Western blot bands and quantification of diaphragm muscles show that the inhibitor $\varepsilon V_{1\text{-}2}$ peptide avoids the phosphorylation induced by the synaptic activity and emphasizes the role of nPKCε. **b** Western blot bands and quantification show that electrical stimulation significantly decreases nPKCε and pnPKCε, especially from the membrane. Data are mean percentage ± SEM, $^*p < 0.05$ ($n = 5$)

47/TrkB in basal conditions increases SNAP-25 (40.00% ± 8.49; $p < 0.05$) and pSNAP-25 (30.41% ± 7.32; $p < 0.05$) without affecting the ratio (− 6.86% ± 3.23; $p > 0.05$). Figure 6b shows that under electrical stimulation, neither 47/TrkB nor BDNF affected SNAP-25, pSNAP-25, or the ratio pSNAP-25/SNAP-25. This result suggests that TrkB does not modulate SNAP-25 phosphorylation under synaptic activity.

## Muscle Contraction Prevents Synaptic Activity-Induced SNAP-25 Phosphorylation at the NMJ

Muscle activity enhances the presynaptic nPKCε and cPKCβI levels through BDNF/TrkB pathway [23, 24, 26]. Because of that, we investigated the role of muscle contraction on SNAP-25 and pSNAP-25 and their relation to nPKCε, cPKCβI, and the BDNF/TrkB signaling.

Figure 7a shows that electrical stimulation with muscle contraction did not change SNAP-25 (1.92% ± 6.9; $p > 0.05$) or pSNAP-25 levels (− 11.28% ± 3.69; $p > 0.05$) when compared to basal conditions. Interestingly, when muscle contraction was compared to stimulated muscles without contraction, we found that muscle contraction significantly decreased pSNAP-25. In accordance, the pSNAP-25/SNAP-25 ratio also decreased (− 17.92% ± 7.28; $p < 0.05$).

We next asked how this condition works at shorter times (Fig. 7b). Results show that after 10 min, SNAP-25 decreased (− 24.36% ± 2.51; $p < 0.05$) and it recovered after 30 min

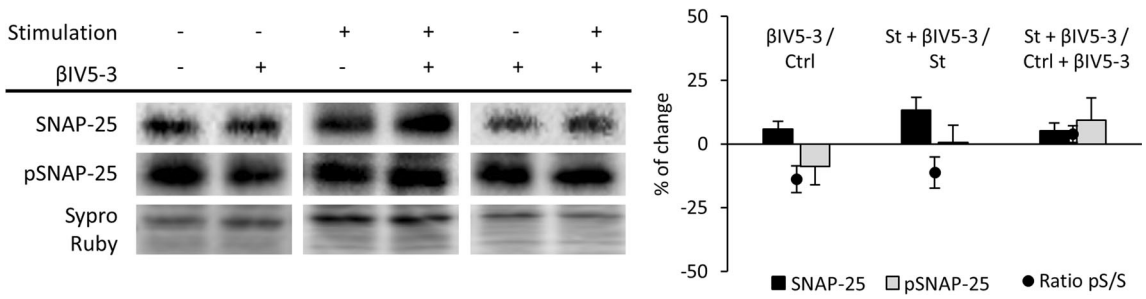



**Fig. 5** cPKCβI does not regulate SNAP-25 phosphorylation. Western blot bands and quantification of diaphragm muscles show that the inhibitor $\beta IV_{5\text{-}3}$ peptide does not affect SNAP-25 or pSNAP-25 under any condition. Data are mean percentage ± SEM, $^*p < 0.05$ ($n = 5$)

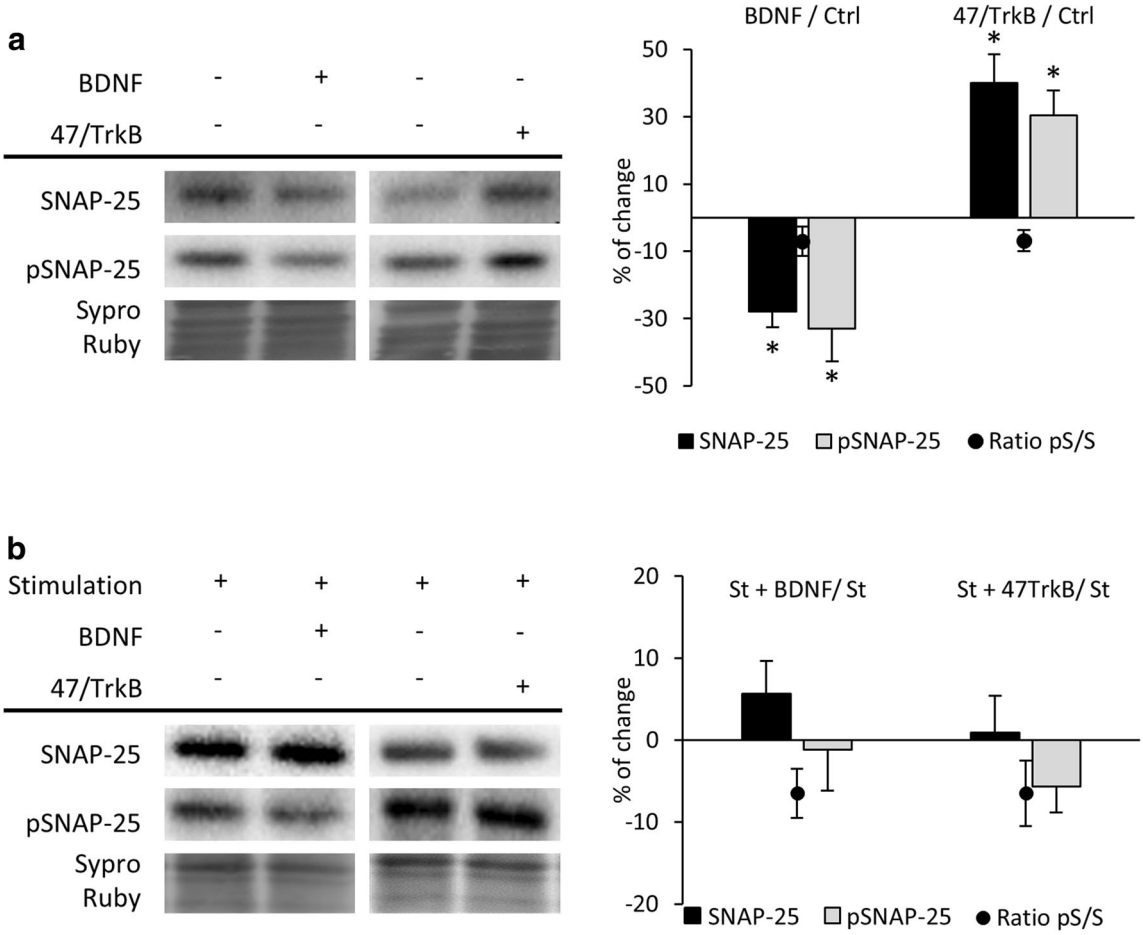



**Fig. 6** *BDNF*/TrkB signaling only modulates SNAP-25 and pSNAP-25 levels under basal conditions. **a** Western blot bands and quantification of diaphragm muscles show that the inhibitor 47/TrkB increases the levels of SNAP-25 and pSNAP-25 while BDNF reduces them in basal conditions. The ratio of pSNAP-25/SNAP-25 does not change. **b** Western blot bands and quantification show that under electrical stimulation, neither 47/TrkB nor BDNF affect SNAP-25 or pSNAP-25. Data are mean percentage ± SEM, $*p < 0.05$ ($n = 3$ in **a**, $n = 5$ in **b**)

while pSNAP-25 did not change in time. Altogether, neuromuscular activity does not change pSNAP-25 because muscle contraction may revert the synaptic activity-induced SNAP-25 phosphorylation.

We also analyzed whether nPKCε is also related to SNAP-25 phosphorylation when nerve stimulation results in muscle contraction by studying the effect of $\varepsilon V_{1\text{-}2}$. We found a significant decrease in pSNAP-25 levels (− 26.55% ± 3.17; $p < 0.05$) and unchanged SNAP-25 (9.07% ± 3.40; $p > 0.05$) (Fig. 7c). In accordance, the ratio of pSNAP-25/SNAP-25 significantly decreased (− 32.65% ± 4.97; $p < 0.05$). This result indicates that nPKCε positively regulates SNAP-25 phosphorylation also in contracting muscles. When contracting muscles were treated with $\beta IV_{5\text{-}3}$, SNAP-25 and pSNAP-25 did not change (Fig. 7c). This result confirms that cPKCβI does not regulate SNAP-25 synthesis or phosphorylation under any condition.

Finally, we analyzed the possible regulation of the BDNF/TrkB signaling pathway. Blocking TrkB with 47/TrkB in contraction did not change SNAP-25 (10.54% ± 5.38; $p > 0.05$) or pSNAP-25 (− 11.31% ± 4.07; $p > 0.05$) (Fig. 7d). In concordance, exogenous BDNF neither changed SNAP-25 (5.36% ± 2.2; $p > 0.05$) nor pSNAP-25 (− 14.53% ± 5.25; $p > 0.05$) (Fig. 7e. Therefore, the BDNF/TrkB signaling does not regulate SNAP-25 nor pSNAP-25 in contracting conditions.

In summary, the phosphorylation of SNAP-25 at the NMJ is increased in response to a signaling mechanism initiated with synaptic activity and directly mediated by nPKCε without the action of cPKCβI and TrkB. Moreover, muscle contraction returns SNAP-25 phosphorylation to the basal situation.

## Discussion

SNAP-25 is a component of the SNARE complex involved with synaptic activity-dependent exocytosis. Its absence completely blocks synaptic transmission due to the emptying of the primed vesicle pools [18]. After the ready releasable pools have been emptied, the PKC phosphorylation on SNAP-

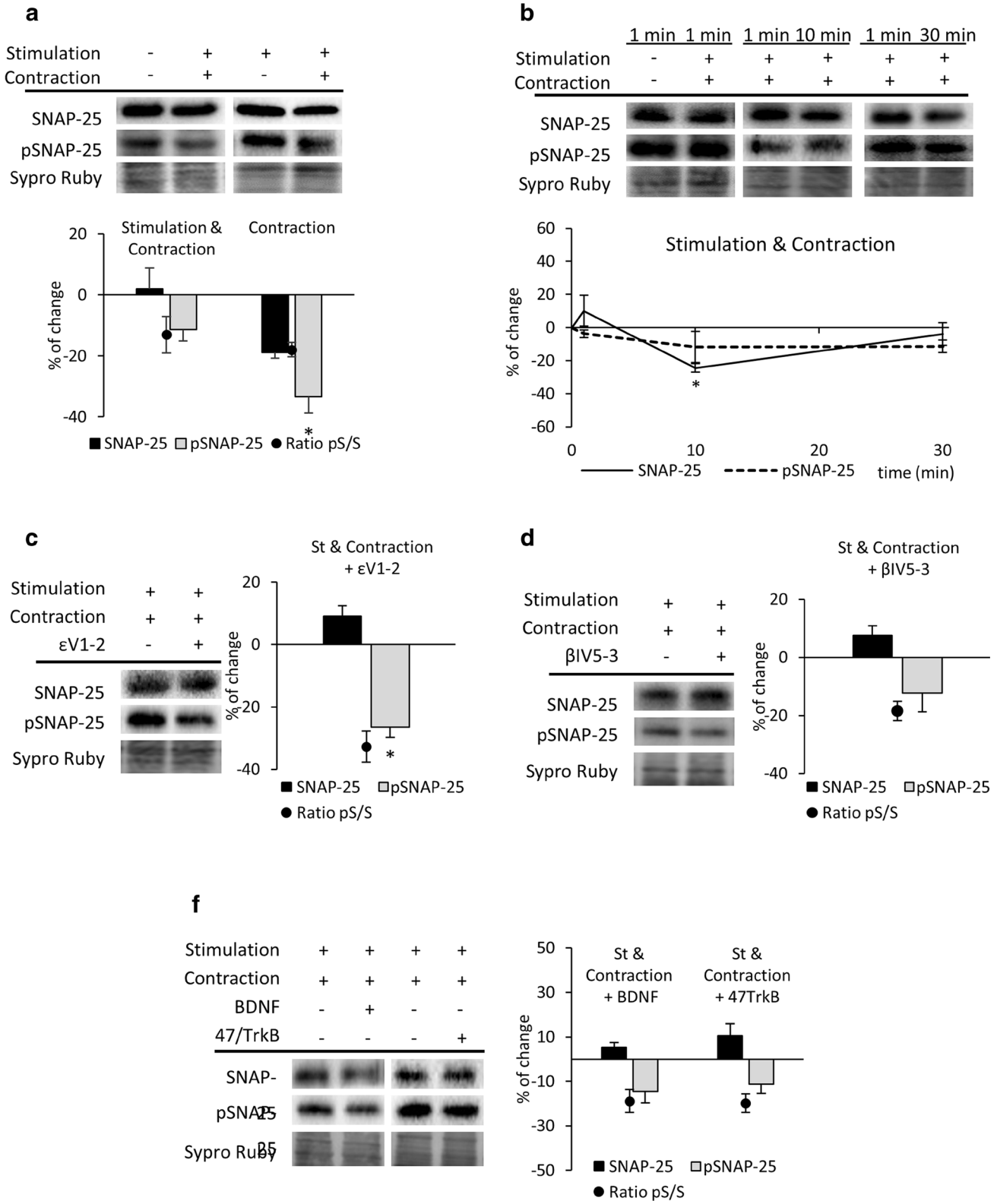



**Fig. 7** nPKCε modulates SNAP-25 phosphorylation under muscle contraction independently from the BDNF/TrkB signaling pathway. **a** Western blot bands and quantification of diaphragm muscles show that electrical stimulation (1 Hz, 30 min) resulting in contraction decreases SNAP-25 phosphorylation when compared to stimulation without contraction. **b** Western blot bands and the time course representation show that muscle contraction decreases SNAP-25 at 10 min and recovers it at 30 min. **c** The $\varepsilon V_{1\text{-}2}$ peptide decreases SNAP-25 phosphorylation and the pSNAP-25/SNAP-25 ratio under stimulation resulting in contraction. **d** The $\beta IV_{5\text{-}3}$ peptide does not affect pSNAP-25 nor SNAP-25 under stimulation resulting in contraction. **e** Neither exogenous BDNF nor 47/TrkB affect pSNAP-25 levels. Data are mean percentage ± SEM, $^*p < 0.05$ ($n = 5$)

25 (Ser-187) regulates the refilling of the synaptic vesicles [15–17]. However, although the PKC action on SNAP-25 has been well characterized, its synaptic location and regulation by PKC isoforms in the NMJ have never been determined. Our results support that the novel PKCε isoform is essential to positively regulate the phosphorylation of SNAP-25 on Ser-187 during synaptic activity and that muscle contraction prevents it. Furthermore, TrkB and cPKCβI activities do not regulate SNAP-25 in activity conditions.

In this study, we report that SNAP-25 expression and phosphorylation at Ser-187 behave differently in the adult rat diaphragm and several brain regions. Previous studies also found that SNAP-25 immunoreactivity is high in the adult rat cortex while generally low or absent in regions with fewer axon terminals like the spinal cord [55–57] or the skeletal muscle [28]. This is because SNAP-25 is preferentially located in the presynaptic terminal, both in central [58–60] and peripheral synapses (present results, [61, 62]). Specifically, SNAP-25 has been found at the active zones of NMJ with dSTORM microscopy [63]. Our results are in accordance with this location. Indeed, evidence shows that SNAP-25 is located at very much lower levels in the postsynaptic terminal [64–68]. Thus, the absence we detect of SNAP-25 in the postsynaptic component of the NMJ is in line with its difficult detection in dendritic spines. Moreover, finding anti-SNAP-25 immunoreactivity predominant in the nervous tissue and exclusive of the presynaptic terminal further discards antibody cross-reactivity with SNAP-23, which is ubiquitously expressed [27, 28] and diffusely located in the cytosol of skeletal muscle cells by immunohistochemistry [29].

## Synaptic Activity Increases pSNAP-25 Through nPKCε

The induction of Ser-187 phosphorylation of SNAP-25 results in its translocation to the plasma membrane in PC12 cells [10]. Here, we show that nerve stimulation for 30 min also increases pSNAP-25 in the membrane fraction at the NMJ. This phosphorylation is likely a slow-adaptive mechanism to cope with long stimulation times, as we found that shorter stimulation times fail to induce it. This could indicate that the basal levels of pSNAP-25 in the membrane are enough for short stimulation times, but sustained activity requires further translocation to the plasma membrane. In concordance, trans-SNARE complexes containing SNAP-25 already exist in the membrane before $Ca^{2+}$ triggering of neurotransmitter release [69]. Also, previous studies report that physiological stimuli induce SNAP-25 phosphorylation in PC12 cells, hippocampal organotypic cultures, and adrenal chromaffin cells [9, 10, 16, 70] to modulate neurotransmission and plasticity [15].

It is well-known that synaptic activity induces PKC action through the second messengers $Ca^{2+}$ and DAG [39, 71–74], which have also been described to increase SNAP-25 phosphorylation in different cells [9, 15, 16]. Here, we report that, at the NMJ, high external calcium increases pSNAP-25 while the absence of calcium by BAPTA-AM reduces it. This is in accordance with previous research where calcium couples PKC isoforms to neurotransmission [3, 23]. Similarly, synaptic stimulation of nerve endings has been reported to induce intracellular calcium entry and SNAP-25 phosphorylation [75] as well as other proteins of the exocytotic machinery, e.g., Munc18-1 and synaptotagmin I [43, 76]. In turn, after nerve stimulation, PKC regulates the activity-dependent release at the NMJ [23–26, 46]. Possibly, this role is in part mediated by the phosphorylation on SNAP-25, which influences the release process by regulating the refilling of synaptic vesicles [15–17].

PKC activation by PMA increases the phosphorylation of SNAP-25 in PC12 cells as well as in hippocampal cultures [9, 21]. In line, we report that PMA induces SNAP-25 phosphorylation in various regions of the central nervous system (CNS), including the hippocampus. Interestingly, PMA was unable to increase pSNAP-25 in the diaphragm muscle. The high quantal release in the NMJ as compared with the small central synapses may explain such functional adaptation [77]. Diaphragm and CNS tissues could be different in PMA-sensitivity, PKC isoform expression and activity, or substrate preference in response to PMA. Also, SNAP-25 is a highly regulated SNARE protein known to be degraded after activity [68, 78]. PMA induces spontaneous release at the NMJ [79] possibly involving SNAP-25. Thus, degradation after activity could be masking the increase in phosphorylation. Alternatively, it could be related to the time course that PMA requires to induce SNAP-25 phosphorylation. On the other hand, PMA could induce SNAP-25 phosphorylation, but a subsequent decrease in the phosphorylation of SNAP-25 could be caused by a phosphatase activity as the action of several phosphatases regulate synaptic activity at the NMJ [24, 43].

Also, we found that PMA enhances SNAP-25 expression in the diaphragm muscle but not in the CNS. Taking into account the timing difference, previous research in a model of heterozygous deletion of SNAP-25 suggested the existence of an adaptative mechanism of SNAP-25 expression in phrenic nerve terminals but absent in the brain [80]. In our model, the potent PKC activator PMA could be affecting or potentiating that mechanism. PMA acts through many intracellular pathways [32, 81, 82], including the increase of protein expression or post-translational changes at a wide range of time (1–48 h) [81, 83–85]. Additionally, the fast nature of the SNAP-25 increase after PMA; it could be related to the existence of SNAP-25 mRNA at the NMJ [86], a functional adaptation to the higher demands of the NMJ due to the larger number of vesicles released per impulse when compared with

central synapses [77], and the essential role of SNAP-25 for neurotransmission.

The nPKCε isoform could be the mediator of PMA effects on SNAP-25 levels. In line, we found that the inhibitor peptide $\varepsilon V_{1\text{-}2}$, which disrupts the nPKCε-RACK1 complex, reduces SNAP-25 levels at basal conditions. This is probably caused by a modulation of protein translation, because SNAP-25 (Fig. 2) and nPKCε [26] are exclusive of the nerve terminal of the NMJ and, in our model, phrenic nerve terminals are separated from their soma. Other studies using PKC inhibitor peptides showed that nPKCε-RACK1 stabilizes mRNA in the CNS [87, 88] and acts directly on the ribosome to induce protein translation [89]. Interestingly, we previously found that nPKCε in basal conditions also enhances the expression of Munc18-1, another molecule related to synaptic release [43].

In addition, nPKCε participates in SNAP-25 phosphorylation during synaptic activity indicating that nPKCε is necessary for synaptic activity to induce SNAP-25 phosphorylation at the NMJ. The presynaptic novel nPKCε and the conventional cPKCβI maintain and potentiate, respectively, the ACh release at the NMJ [24, 25] and, because of that, both could be candidates to phosphorylate SNAP-25. Here, we have determined that, among the two PKC isoforms studied, cPKCβI does not contribute to SNAP-25 phosphorylation and only nPKCε regulates the phosphorylation of SNAP-25 during synaptic activity at the NMJ. This coincides with a reduction in nPKCε and pnPKCε probably due to consumption after its catalytic activity [26, 50, 53, 90, 91]. The turnover is also reflected in the decrease of nPKCε and pnPKCε in the membrane (Fig. 4b), where PKC is active, indicating the presence of a functional nPKCε pool since the beginning of synaptic activity (and even in basal conditions). Blocking nPKCε translocation to the membrane in electrophysiological experiments has revealed that this isoform controls ACh release at the NMJ [25] and enhances Munc18-1 phosphorylation [43]. Therefore, this PKC isoform has a key role in regulating the SNARE/SM complex and exocytosis during synaptic activity.

In comparison with prior studies, the PKC phosphorylation of SNAP-25 behaves similarly to that of Munc18-1 after high $Ca^{2+}$ and nerve-induced stimulation at the NMJ [43]. This could be due to their shared activity in the SNARE/SM complex formation to guarantee an appropriate synaptic vesicle exocytosis [69]. However, SNAP-25 and Munc18-1 respond differently when PKC is modulated through the phorbol ester PMA. Here, we determined that PMA increases the total protein level of SNAP-25 but not its phosphorylation. Maybe, the phosphorylating effect by PMA is masked by an increased turnover and degradation of pSNAP-25, which is prominent in the membrane fraction at basal conditions. Interestingly, PMA increases both total and phosphorylated Munc18-1 [43]. This difference between Munc18-1 and SNAP-25 phosphorylation could be important for the regulation of the synaptic vesicle exocytosis. Several mechanisms could explain this outcome. For instance, SNAP-25 and Munc18-1 are regulated by different PKC isoforms. In particular, Munc18-1 is modulated by both cPKCβI and nPKCε [43] while SNAP-25 relies only on nPKCε. In relation to cPKCβI, it does not contribute to SNAP-25 synthesis or phosphorylation during synaptic activity, contrary to the negative role that it has over Munc18-1 [43]. Also, it is known that phorbol esters affect neurotransmission directly through Munc13 [32, 82].

In summary, all these data indicate that pSNAP-25 levels are high enough in the membrane in basal conditions to allow its function during the initial period of activity. Moreover, PMA stimulation of PKC does not increase pSNAP-25, which suggests that there is enough amounts of it. Finally, continued electrical stimulation of the NMJ increases pSNAP-25 in the membrane after 30 min, probably through nPKCε isoform but independently from cPKCβI.

## Muscle Contraction Prevents SNAP-25 Phosphorylation Without BDNF/TrkB Signaling

The present work shows that muscle contraction per se executes a retrograde control over the presynaptic terminal to guarantee a sustained pool of pSNAP-25. This could modulate the action on pSNAP-25 induced by nerve stimulation and allow the appropriate refilling of the synaptic vesicle pools [15–17]. Additionally, pSNAP-25 reduction could also affect the negative activity of pSNAP-25 on the VGCC channels [21]. This feedback could be in relation to the extended control of the presynaptic activity as the negative action of the $M_2$ mAChR and $A_1$ adenosine receptor on transmitter release [92–94].

Skeletal muscle cells are metabolically active and communicate through active secretion of a wide variety of neurotrophic factors, cytokines, myokines, and other substances that exert auto-, para-, and endocrine effects in response to contraction. These factors not only regulate energy demand but also improve health and coordinate the control of motor neuron innervation and the neuromuscular junction formation and maintenance. Our previous results showed that increased BDNF by muscle contraction retrogradely enhances presynaptic nPKCε and cPKCβI levels [23, 24, 26]. However, this neurotrophic factor does not regulate SNAP-25 phosphorylation suggesting that another signal derived from the muscle cell is involved. The bidirectional interaction between muscles and the nervous system is fundamental for their health and future studies on them could help to further identify the mechanism that induces the negative feedback of muscle activity not only on SNAP-25 phosphorylation but also in other presynaptic elements.

In this condition, where pSNAP-25 levels are closely controlled, nPKCε keeps upregulating them. The fact that nPKCε

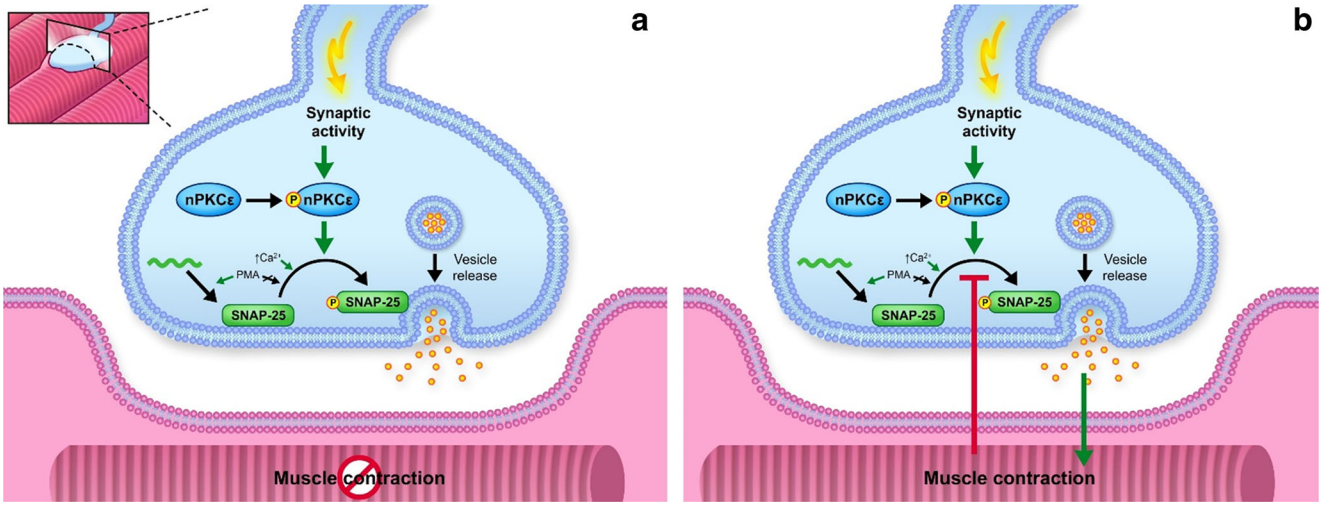



**Fig. 8** Summary. **a** SNAP-25 is expressed and phosphorylated in basal conditions in the skeletal muscle. It is placed in the motor nerve terminals and absent in the Schwann cells and muscle cells. SNAP-25 phosphorylation at the residue Ser-187 occurs in response to PKC activation, specifically nPKCε, by high extracellular $Ca^{2+}$ and electrical stimulation, but not by PMA. **b** Muscle contraction prevents the synaptic activity-induced SNAP-25 phosphorylation

has a consistent phosphorylating action in all studied conditions—contrary to cPKCβI—confirms the importance of the novel isoform in the regulation of SNAP-25. Interestingly, although muscular activity enhances the presynaptic nPKCε through the BDNF/TrkB signaling [26, 43], this receptor does not affect SNAP-25. The fraction of increased nPKCε might preferentially phosphorylate other substrates, such as pMARCKS, which increases during contraction through the TrkB signaling [26], because of the demands of muscle activity. The positive action of nPKCε on SNAP-25 could be modulated by other metabotropic receptors present at the NMJ like the muscarinic and purinergic receptors. These are coupled to the PLC beta isoform [95–97], which is different from the PLC gamma isoform coupled to TrkB [98]. On the other hand, it has been shown that neurotrophin-4/5 (NT-4/5) acts differently from BDNF, maintaining a longer sustained downstream signaling activation of TrkB [99]. This could be in line with the slow-adaptative of SNAP-25 phosphorylation that we observed. Therefore, studies on other neurotrophins and metabotropic pathways could help to further identify the activity-dependent action of nPKCε on SNAP-25 and maybe also the mechanism that induces the negative feedback of postsynaptic activity on SNAP-25 phosphorylation induced by nPKCε.

Regarding BDNF and TrkB, the decrease in SNAP-25 and pSNAP-25 induced by exogenous BDNF through TrkB in basal conditions suggests that this neurotrophin mimics the effect of muscle contraction to control SNAP-25 levels. This is in accordance with the fact that TrkB inhibition impairs the release in NMJ [100, 101]. It is interesting how the effect of both exogenous BDNF and 47/TrkB on SNAP-25 disappears when neuromuscular activity is present. The change between basal and activity conditions could be important to control the amount of SNAP-25 at the nerve terminal. Moreover, endogenous BDNF could decrease SNAP-25 by stimulating proteases [78, 102] or by inhibiting the translation of SNAP-25 [86] to maintain the protein pool. This action would counterbalance the positive action of nPKCε over SNAP-25 in basal conditions.

Moreover, muscle contraction decreases SNAP-25 at short time of stimulation. SNAP-25 decreases at 10 min maybe because of consume-dependent degradation as it is known that the stability of SNAP-25 at the presynaptic component is controlled by activity-induced ubiquitination and proteasome-dependent degradation [68, 78]. However, it is recovered at 30 min, when the retrograde effect of muscle contraction restores the protein local pool to maintain the protein levels and guarantee the release in the system in case of prolonged demand.

## Concluding Remarks

The phosphorylation of SNAP-25 is essential for neurotransmitter secretion. Figure 8 summarizes the proposed mechanism that regulates SNAP-25 phosphorylation at the nerve terminals of the NMJ. In brief, pSNAP-25 is enhanced by synaptic activity and translocated to the membrane. This is mediated by nPKCε in activity conditions without the participation of cPKCβI and BDNF/TrkB signaling. As a feedback, muscle contraction returns pSNAP-25 to the basal levels, indicating that nerve terminals need both pre- and postsynaptic activities to modulate SNAP-25 phosphorylation and ensure an accurate neurotransmission process.

**Acknowledgments** We would like to thank Dr. Daria Mochly-Rosen and Dr. Nir Qvit for providing the specific translocation inhibitor peptide of cPKCβI ($\beta IV_{5-3}$).

**Authors' Contributions** A.S.: data collection, quantitative analysis, literature search, data interpretation, statistics; V.C., L.J.: data collection, quantitative analyses, literature search, data interpretation, design graphic abstract; E.H., L.N., and M.T.: data interpretation; J.T., M.A.L, and N.G.: conception and design, literature search, data interpretation, manuscript preparation

**Funding Information** This work has been possible with the support of the Ministerio de Economía, Industria y Competitividad (MINECO), the Agencia Estatal de Investigación (AEI), the European Regional Development Fund (ERDF) (SAF2015-67143-P), the Universitat Rovira i Virgili (URV) (2014PFR-URV-B2-83 and 2017PFR-URV-B2-85), and the Catalan Government (2014SGR344 and 2017SGR704). V.C. has been supported by MINECO under the framework of the Sistema Nacional de Garantía Juvenil, the European Social Fund (ESF), and the Iniciativa de Empleo Juvenil (IEJ).

## Compliance with Ethical Standards

Young adult Sprague-Dawley rats (30–40 days; Criffa, Barcelona, Spain; RRID:RGD_5508397) were cared for in accordance with the guidelines of the European Community Council Directive for the humane treatment of laboratory animals. All the procedures were approved by the Animal Experimentation Ethics Committee of the Universitat Rovira i Virgili.

**Competing Interests** The authors declare that they have no competing interests.



## References

1. Byrne JH, Kandel ER (1996) Presynaptic facilitation revisited: state and time dependence. J Neurosci 16:425–435. https://doi.org/10.1523/JNEUROSCI.16-02-00425
2. Numann R, Hauschka SD, Catterall WA, Scheuer T (1994) Modulation of skeletal muscle sodium channels in a satellite cell line by protein kinase C. J Neurosci 14:4226–4236. https://doi.org/10.1523/JNEUROSCI.14-07-04226
3. Santafé MM, Lanuza MA, Garcia N, Tomàs J (2005) Calcium inflow-dependent protein kinase C activity is involved in the modulation of transmitter release in the neuromuscular junction of the adult rat. Synapse 57:76–84. https://doi.org/10.1002/syn.20159
4. Santafé MM, Lanuza MA, Garcia N, Tomàs J (2006) Muscarinic autoreceptors modulate transmitter release through protein kinase C and protein kinase A in the rat motor nerve terminal. Eur J Neurosci 23:2048–2056. https://doi.org/10.1111/j.1460-9568.2006.04753.x
5. Tomàs J, Santafé MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Obis T, Priego M et al (2014) Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. J Neurosci Res 92:543–554. https://doi.org/10.1002/jnr.23346
6. West J, Numann R, Murphy B et al (1991) Identification of an intracellular peptide segment involved in sodium channel inactivation. Science (80- ) 241:1658–1661. https://doi.org/10.1126/science.2458625
7. Catterall WA (1999) Interactions of presynaptic Ca2+ channels and Snare proteins in neurotransmitter release. Ann N Y Acad Sci 868:144–159. https://doi.org/10.1111/j.1749-6632.1999.tb11284.x
8. Snyder DA, Kelly ML, Woodbury DJ (2006) SNARE complex regulation by phosphorylation. Cell Biochem Biophys 45:111–123. https://doi.org/10.1385/CBB:45:1:111
9. Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, Takahashi M (1996) Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. J Biol Chem 271:14548–14553. https://doi.org/10.1074/JBC.271.24.14548
10. Kataoka M, Kuwahara R, Iwasaki S, Shoji-Kasai Y, Takahashi M (2000) Nerve growth factor-induced phosphorylation of SNAP-25 in PC12 cells. J Neurochem 74:2058–2066. https://doi.org/10.1046/j.1471-4159.2000.0742058.x
11. Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318–324. https://doi.org/10.1038/362318a0
12. Mohrmann R, de Wit H, Verhage M, Neher E, Sorensen JB (2010) Fast vesicle fusion in living cells requires at least three SNARE complexes. Science 330:502–505. https://doi.org/10.1126/science.1193134
13. Morgan A, Burgoyne RD, Barclay JW, Craig TJ, Prescott GR, Ciufo LF, Evans GJO, Graham ME (2005) Regulation of exocytosis by protein kinase C. Biochem Soc Trans 33:1341–1344. https://doi.org/10.1042/BST20051341
14. Yang Y, Craig TJ, Chen X, Ciufo LF, Takahashi M, Morgan A, Gillis KD (2007) Phosphomimetic mutation of Ser-187 of SNAP-25 increases both syntaxin binding and highly Ca2+-sensitive exocytosis. J Gen Physiol 129:233–244. https://doi.org/10.1085/jgp.200609685
15. Katayama N, Yamamori S, Fukaya M, Kobayashi S, Watanabe M, Takahashi M, Manabe T (2017) SNAP-25 phosphorylation at Ser187 regulates synaptic facilitation and short-term plasticity in an age-dependent manner. Sci Rep 7:7996. https://doi.org/10.1038/s41598-017-08237-x
16. Nagy G, Matti U, Nehring RB, Binz T, Rettig J, Neher E, Sørensen JB (2002) Protein kinase C-dependent phosphorylation of synaptosome-associated protein of 25 kDa at Ser187 potentiates vesicle recruitment. J Neurosci 22:9278–9286. https://doi.org/10.1523/JNEUROSCI.22-21-09278.2002
17. Leenders AGM, Sheng Z-H (2005) Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. Pharmacol Ther 105:69–84. https://doi.org/10.1016/j.pharmthera.2004.10.012
18. Sørensen JB, Nagy G, Varoqueaux F, Nehring RB, Brose N, Wilson MC, Neher E (2003) Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. Cell 114:75–86. https://doi.org/10.1016/S0092-8674(03)00477-X
19. Sørensen JB, Matti U, Wei S-H et al (2002) The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. Proc Natl Acad Sci U S A 99:1627–1632. https://doi.org/10.1073/pnas.251673298
20. Zhang Z, Wang D, Sun T, Xu J, Chiang HC, Shin W, Wu LG (2013) The SNARE proteins SNAP25 and synaptobrevin are involved in endocytosis at hippocampal synapses. J Neurosci 33:9169–9175. https://doi.org/10.1523/JNEUROSCI.0301-13.2013
21. Pozzi D, Condliffe S, Bozzi Y, Chikhladze M, Grumelli C, Proux-Gillardeaux V, Takahashi M, Franceschetti S et al (2008) Activity-dependent phosphorylation of Ser187 is required for SNAP-25-negative modulation of neuronal voltage-gated calcium channels. Proc Natl Acad Sci U S A 105:323–328. https://doi.org/10.1073/pnas.0706211105

22. Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T et al (2004) SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. Neuron 41:599–610. https://doi.org/10.1016/S0896-6273(04)00077-7
23. Besalduch N, Tomàs M, Santafé MM et al (2010) Synaptic activity-related classical protein kinase C isoform localization in the adult rat neuromuscular synapse. J Comp Neurol 518:211–228. https://doi.org/10.1002/cne.22220
24. Hurtado E, Cilleros V, Nadal L et al (2017) Muscle contraction regulates BDNF/TrkB signaling to modulate synaptic function through presynaptic cPKCα and cPKCβI. Front Mol Neurosci 10:1–22. https://doi.org/10.3389/fnmol2017.00147
25. Obis T, Hurtado E, Nadal L, Tomàs M, Priego M, Simon A, Garcia N, Santafe MM et al (2015) The novel protein kinase C epsilon isoform modulates acetylcholine release in the rat neuromuscular junction. Mol Brain 8:80. https://doi.org/10.1186/s13041-015-0171-5
26. Obis T, Besalduch N, Hurtado E, Nadal L, Santafe MM, Garcia N, Tomàs M, Priego M et al (2015) The novel protein kinase C epsilon isoform at the adult neuromuscular synapse: location, regulation by synaptic activity-dependent muscle contraction through TrkB signaling and coupling to ACh release. Mol Brain 8:8. https://doi.org/10.1186/s13041-015-0098-x
27. Ravichandran V, Chawla A, Roche PA (1996) Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. J Biol Chem 271:13300–13303. https://doi.org/10.1074/JBC.271.23.13300
28. Yu Y, Fuscoe JC, Zhao C, Guo C, Jia M, Qing T, Bannon DI, Lancashire L et al (2014) A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages. Nat Commun 5:3230. https://doi.org/10.1038/ncomms4230
29. Boström P, Andersson L, Vind B, Håversen L, Rutberg M, Wickström Y, Larsson E, Jansson PA et al (2010) The SNARE protein SNAP23 and the SNARE-interacting protein Munc18c in human skeletal muscle are implicated in insulin resistance/type 2 diabetes. Diabetes 59:1870–1878. https://doi.org/10.2337/db09-1503
30. Rapaport D, Lugassy Y, Sprecher E, Horowitz M (2010) Loss of SNAP29 impairs endocytic recycling and cell motility. PLoS One 5:e9759. https://doi.org/10.1371/journal.pone.0009759
31. Iwasaki S, Kataoka M, Sekiguchi M, Shimazaki Y, Sato K, Takahasi M (2000) Two distinct mechanisms underlie the stimulation of neurotransmitter release by phorbol esters in clonal rat pheochromocytoma PC12 cells. J Biochem 128:407–414
32. Wierda KDB, Toonen RFG, de Wit H, Brussaard AB, Verhage M (2007) Interdependence of PKC-dependent and PKC-independent pathways for presynaptic plasticity. Neuron 54:275–290. https://doi.org/10.1016/j.neuron.2007.04.001
33. Losavio A, Muchnik S (2000) Facilitation of spontaneous acetylcholine release induced by activation of cAMP in rat neuromuscular junctions. Life Sci 66:2543–2556. https://doi.org/10.1016/S0024-3205(00)00588-9
34. Liu GS, Cohen MV, Mochly-Rosen D, Downey JM (1999) Protein kinase C- ξ is responsible for the protection of preconditioning in rabbit cardiomyocytes. J Mol Cell Cardiol 31:1937–1948. https://doi.org/10.1006/jmcc.1999.1026
35. Zhang Y, Ying J, Jiang D, Chang Z, Li H, Zhang G, Gong S, Jiang X et al (2015) Urotensin-II receptor stimulation of cardiac L-type Ca2+ channels requires the βγ subunits of Gi/o-protein and phosphatidylinositol 3-kinase-dependent protein kinase C β1 isoform. J Biol Chem 290:8644–8655. https://doi.org/10.1074/jbc.M114.615021
36. Johnson JA, Gray MO, Chen CH, Mochly-Rosen D (1996) A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. J Biol Chem 271:24962–24966. https://doi.org/10.1074/JBC.271.40.24962
37. Stebbins EG, Mochly-Rosen D (2001) Binding specificity for RACK1 resides in the V5 region of beta II protein kinase C. J Biol Chem 276:29644–29650. https://doi.org/10.1074/jbc.M101044200
38. Stebbins EG, Mochly-Rosen D (2001) Binding specificity for RACK1 resides in the V5 region of βII protein kinase C. J Biol Chem 276:29644–29650. https://doi.org/10.1074/jbc.M101044200
39. Mochly-Rosen D, Gordon AS (1998) Anchoring proteins for protein kinase C: a means for isozyme selectivity. FASEB J 12:35–42. https://doi.org/10.1096/fasebj.12.1.35
40. Way KJ, Chou E, King GL (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. Trends Pharmacol Sci 21:181–187. https://doi.org/10.1016/S0165-6147(00)01468-1
41. Di-Capua N, Sperling O, Zoref-Shani E (2003) Protein kinase C-ε is involved in the adenosine-activated signal transduction pathway conferring protection against ischemia-reperfusion injury in primary rat neuronal cultures. J Neurochem 84:409–412. https://doi.org/10.1046/j.1471-4159.2003.01563.x
42. Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO et al (1999) A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. Neuron 24:253–260. https://doi.org/10.1016/S0896-6273(00)80837-5
43. Simó A, Just-Borràs L, Cilleros-Mañé V, Hurtado E, Nadal L, Tomàs M, Garcia N, Lanuza MA et al (2018) 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 11:207. https://doi.org/10.3389/fnmol.2018.00207
44. Cazorla M, Prémont J, Mann A, Girard N, Kellendonk C, Rognan D (2011) Identification of a low–molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice. J Clin Invest 121:1846–1857. https://doi.org/10.1172/JCI43992
45. Favreau P, Le Gall F, Benoit E, Molgó J (1999) A review on conotoxins targeting ion channels and acetylcholine receptors of the vertebrate neuromuscular junction. Acta Physiol Pharmacol Ther Latinoam 49:257–267
46. Santafé MM, Garcia N, Lanuza MA, Tomàs M, Tomàs J (2009) Interaction between protein kinase C and protein kinase A can modulate transmitter release at the rat neuromuscular synapse. J Neurosci Res 87:683–690. https://doi.org/10.1002/jnr.21885
47. Lau CG, Takayasu Y, Rodenas-Ruano A, Paternain AV, Lerma J, Bennett MVL, Zukin RS (2010) SNAP-25 is a target of protein kinase C phosphorylation critical to NMDA receptor trafficking. J Neurosci 30:242–254. https://doi.org/10.1523/JNEUROSCI.4933-08.2010
48. Zhang ZH, Johnson JA, Chen L, el-Sherif N, Mochly-Rosen D, Boutjdir M (1997) C2 region-derived peptides of beta-protein kinase C regulate cardiac Ca2+ channels. Circ Res 80:720–729. https://doi.org/10.1161/res.80.5.720
49. Parker PJ, Bosca L, Dekker L, Goode NT, Hajibagheri N, Hansra G (1995) Protein kinase C (PKC)-induced PKC degradation: a model for down-regulation. Biochem Soc Trans 23:153–155. https://doi.org/10.1042/BST0230153
50. Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA (1998) Activation of protein kinase C triggers its ubiquitination and degradation. Mol Cell Biol 18:839–845. https://doi.org/10.1128/MCB.18.2.839
51. Lehel C, Oláh Z, Jakab G, Szállási Z, Petrovics G, Harta G, Blumberg PM, Anderson WB (1995) Protein kinase C epsilon subcellular localization domains and proteolytic degradation sites. A model for protein kinase C conformational changes. J Biol

Chem 270:19651–19658. https://doi.org/10.1074/JBC.270.33.19651
52. Olivier AR, Parker PJ (1994) Bombesin, platelet-derived growth factor, and diacylglycerol induce selective membrane association and down-regulation of protein kinase C isotypes in Swiss 3T3 cells. J Biol Chem 269:2758–2763
53. Gould C, Newton A (2008) The life and death of protein kinase C. Curr Drug Targets 9:614–625. https://doi.org/10.2174/138945008785132411
54. Balkowiec A, Katz DM (2000) Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. J Neurosci 20:7417–7423. https://doi.org/10.1523/JNEUROSCI.20-19-07417.2000
55. Oyler GA, Polli JW, Higgins GA, Wilson MC, Billingsley ML (1992) Distribution and expression of SNAP-25 immunoreactivity in rat brain, rat PC-12 cells and human SMS-KCNR neuroblastoma cells. Dev Brain Res 65:133–146. https://doi.org/10.1016/0165-3806(92)90172-S
56. Chen D, Minger SL, Honer WG, Whiteheart S (1999) Organization of the secretory machinery in the rodent brain: distribution of the t-SNAREs, SNAP-25 and SNAP-23. Brain Res 831:11–24. https://doi.org/10.1016/S0006-8993(99)01371-2
57. Mandolesi G, Vanni V, Cesa R, Grasselli G, Puglisi F, Cesare P, Strata P (2009) Distribution of the SNAP25 and SNAP23 synaptosomal-associated protein isoforms in rat cerebellar cortex. Neuroscience 164:1084–1096. https://doi.org/10.1016/J.NEUROSCIENCE.2009.08.067
58. Oyler GA (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol 109:3039–3052. https://doi.org/10.1083/jcb.109.6.3039
59. Holderith N, Lorincz A, Katona G, Rózsa B, Kulik A, Watanabe M, Nusser Z (2012) Release probability of hippocampal glutamatergic terminals scales with the size of the active zone. Nat Neurosci 15:988–997. https://doi.org/10.1038/nn.3137
60. Kerti K, Lorincz A, Nusser Z (2012) Unique somato-dendritic distribution pattern of Kv4.2 channels on hippocampal CA1 pyramidal cells. Eur J Neurosci 35:66–75. https://doi.org/10.1111/j.1460-9568.2011.07907.x
61. Duc C, Catsicas S (1995) Ultrastructural localization of SNAP-25 within the rat spinal cord and peripheral nervous system. J Comp Neurol 356:152–163. https://doi.org/10.1002/cne.903560111
62. Ovespian SV, Bodeker M, O'Leary VB, Lawrence GW, Oliver Dolly J (2015) Internalization and retrograde axonal trafficking of tetanus toxin in motor neurons and trans-synaptic propagation at central synapses exceed those of its C-terminal-binding fragments. Brain Struct Funct 220:1825–1838. https://doi.org/10.1007/s00429-015-1004-0
63. Jones RA, Harrison C, Eaton SL, Llavero Hurtado M, Graham LC, Alkhammash L, Oladiran OA, Gale A et al (2017) Cellular and molecular anatomy of the human neuromuscular junction. Cell Rep 21:2348–2356. https://doi.org/10.1016/j.celrep.2017.11.008
64. Selak S, Paternain AV, Aller IM, Picó E, Rivera R, Lerma J (2009) A role for SNAP25 in internalization of kainate receptors and synaptic plasticity. Neuron 63:357–371. https://doi.org/10.1016/j.neuron.2009.07.017
65. Tomasoni R, Repetto D, Morini R, Elia C, Gardoni F, di Luca M, Turco E, Defilippi P et al (2013) SNAP-25 regulates spine formation through postsynaptic binding to p140Cap. Nat Commun 4:2136. https://doi.org/10.1038/ncomms3136
66. Fossati G, Morini R, Corradini I, Antonucci F, Trepte P, Edry E, Sharma V, Papale A et al (2015) Reduced SNAP-25 increases PSD-95 mobility and impairs spine morphogenesis. Cell Death Differ 22:1425–1436. https://doi.org/10.1038/cdd.2014.227
67. Tao-Cheng JH, Du J, McBain CJ (2000) SNAP-25 is polarized to axons and abundant along the axolemma: an immunogold study of intact neurons. J Neurocytol 29:67–77. https://doi.org/10.1023/A:1007168231323
68. Sharma M, Burré J, Bronk P, Zhang Y, Xu W, Südhof TC (2012) CSPα knockout causes neurodegeneration by impairing SNAP-25 function. EMBO J 31:829–841. https://doi.org/10.1038/emboj.2011.467
69. Pertsinidis A, Mukherjee K, Sharma M, Pang ZP, Park SR, Zhang Y, Brunger AT, Sudhof TC et al (2013) Ultrahigh-resolution imaging reveals formation of neuronal SNARE/Munc18 complexes in situ. Proc Natl Acad Sci U S A 110:E2812–E2820. https://doi.org/10.1073/pnas.1310654110
70. Genoud S, Pralong W, Riederer BM, Eder L, Catsicas S, Muller D (1999) Activity-dependent phosphorylation of SNAP-25 in hippocampal organotypic cultures. J Neurochem 72:1699–1706. https://doi.org/10.1046/j.1471-4159.1999.721699.x
71. Tanaka C, Nishizuka Y (1994) The protein kinase C family for neuronal signaling. Annu Rev Neurosci 17:551–567. https://doi.org/10.1146/annurev.ne.17.030194.003003
72. Steinberg SF (2008) Structural basis of protein kinase C isoform function. Physiol Rev 88:1341–1378. https://doi.org/10.1152/physrev.00034.2007
73. Wang QJ (2006) PKD at the crossroads of DAG and PKC signaling. Trends Pharmacol Sci 27:317–323. https://doi.org/10.1016/j.tips.2006.04.003
74. Osto E, Kouroedov A, Mocharla P, Akhmedov A, Besler C, Rohrer L, von Eckardstein A, Iliceto S et al (2008) Inhibition of protein kinase Cβ prevents foam cell formation by reducing scavenger receptor A expression in human macrophages. Circulation 118:2174–2182. https://doi.org/10.1161/CIRCULATIONAHA.108.789537
75. Südhof TC (2012) Calcium control of neurotransmitter release. Cold Spring Harb Perspect Biol 4:a011353. https://doi.org/10.1101/cshperspect.a011353
76. de Jong APH, Meijer M, Saarloos I, Cornelisse LN, Toonen RFG, Sørensen JB, Verhage M (2016) Phosphorylation of synaptotagmin-1 controls a post-priming step in PKC-dependent presynaptic plasticity. Proc Natl Acad Sci 113:5095–5100. https://doi.org/10.1073/pnas.1522927113
77. Wood SJ, Slater CR (2001) Safety factor at the neuromuscular junction. Prog Neurobiol 64:393–429
78. Sheehan P, Zhu M, Beskow A, Vollmer C, Waites CL (2016) Activity-dependent degradation of synaptic vesicle proteins requires Rab35 and the ESCRT pathway. J Neurosci 36:8668–8686. https://doi.org/10.1523/JNEUROSCI.0725-16.2016
79. Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J (2003) Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. Eur J Neurosci 17:119–127
80. Adler M, Sheridan RE, Deshpande SS, Oyler GA (2001) Neuromuscular transmission and muscle contractility in SNAP-25-deficient coloboma mice. Neurotoxicology 22:775–786. https://doi.org/10.1016/S0161-813X(01)00066-3
81. Huang C-C, Yang D-M, Lin C-C, Kao L-S (2011) Involvement of Rab3A in vesicle priming during exocytosis: interaction with Munc13-1 and Munc18-1. Traffic 12:1356–1370. https://doi.org/10.1111/j.1600-0854.2011.01237.x
82. Rhee JS, Betz A, Pyott S, Reim K, Varoqueaux F, Augustin I, Hesse D, Südhof TC et al (2002) Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. Cell 108:121–133. https://doi.org/10.1016/S0092-8674(01)00635-3
83. Derventzi A, Rattan SI, Clark BF (1993) Phorbol ester PMA stimulates protein synthesis and increases the levels of active elongation factors EF-1 alpha and EF-2 in ageing human fibroblasts.

Mech Ageing Dev 69:193–205. https://doi.org/10.1016/0047-6374(93)90023-K
84. Takagi Y (2004) Phorbol 12-myristate 13-acetate protects Jurkat cells from methylglyoxal-induced apoptosis by preventing c-Jun N-terminal kinase-mediated leakage of cytochrome c in an extracellular signal-regulated kinase-dependent manner. Mol Pharmacol 65:778–787. https://doi.org/10.1124/mol.65.3.778
85. Ballif BA, Blenis J (2001) Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ 12:397–408
86. Islamov RR, Samigullin DV, Rizvanov AA, Bondarenko NI, Nikolskiy EE (2015) Synaptosome-associated protein 25 (SNAP25) synthesis in terminal buttons of mouse motor neuron. Dokl Biochem Biophys 464:272–274. https://doi.org/10.1134/S1607672915050026
87. Alkon DL, Epstein H, Kuzirian A, Bennett MC, Nelson TJ (2005) Protein synthesis required for long-term memory is induced by PKC activation on days before associative learning. Proc Natl Acad Sci U S A 102:16432–16437. https://doi.org/10.1073/pnas.0508001102
88. Hongpaisan J, Xu C, Sen A, Nelson TJ, Alkon DL (2013) PKC activation during training restores mushroom spine synapses and memory in the aged rat. Neurobiol Dis 55:44–62. https://doi.org/10.1016/j.nbd.2013.03.012
89. Larburu N, Montellese C, O'Donohue M-F et al (2016) Structure of a human pre-40S particle points to a role for RACK1 in the final steps of 18S rRNA processing. Nucleic Acids Res 44:8465–8478. https://doi.org/10.1093/nar/gkw714
90. Lee H-W, Smith L, Pettit GR, Vinitsky A, Smith JB (1996) Ubiquitination of protein kinase C-alpha and degradation by the proteasome. J Biol Chem 271:20973–20976. https://doi.org/10.1074/jbc.271.35.20973
91. Kang BS, French OG, Sando JJ, Hahn CS (2000) Activation-dependent degradation of protein kinase C eta. Oncogene 19:4263–4272. https://doi.org/10.1038/sj.onc.1203779
92. Garcia N, Priego M, Obis T, Santafe MM, Tomàs M, Besalduch N, Lanuza MA, Tomàs J (2013) Adenosine A1 and A2A receptor-mediated modulation of acetylcholine release in the mice neuromuscular junction. Eur J Neurosci 38:2229–2241
93. Santafé MM, Priego M, Obis T et al (2015) Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse. Eur J Neurosci 42:1775–1787. https://doi.org/10.1111/ejn.12922
94. Tomàs J, Garcia N, Lanuza MA, Santafé MM, Tomàs M, Nadal L, Hurtado E, Simó-Ollé A et al (2018) Adenosine receptors in developing and adult mouse neuromuscular junctions and functional links with other metabotropic receptor. Pathways 9:1–10. https://doi.org/10.3389/fphar.2018.00397
95. Ross EM, Berstein G (1993) Regulation of the M1 muscarinic receptor-Gq-phospholipase C-beta pathway by nucleotide exchange and GTP hydrolysis. Life Sci 52:413–419. https://doi.org/10.1016/0024-3205(93)90296-F
96. Biddlecome GH, Berstein G, Ross EM (1996) 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 271:7999–8007. https://doi.org/10.1074/JBC.271.14.7999
97. Strassheim D, Williams CL (2000) P2Y2 purinergic and M3 muscarinic acetylcholine receptors activate different phospholipase C-beta isoforms that are uniquely susceptible to protein kinase C-dependent phosphorylation and inactivation. J Biol Chem 275:39767–39772. https://doi.org/10.1074/jbc.M007775200
98. Middlemas DS, Meisenhelder J, Hunter T (1994) Identification of TrkB autophosphorylation sites and evidence that phospholipase C-gamma 1 is a substrate of the TrkB receptor. J Biol Chem 269:5458–5466
99. Proenca CC, Song M, Lee FS (2016) Differential effects of BDNF and neurotrophin 4 (NT4) on endocytic sorting of TrkB receptors. J Neurochem 138:397–406. https://doi.org/10.1111/jnc.13676
100. Garcia N, Tomàs M, Santafé MM et al (2010) Localization of brain-derived neurotrophic factor, neurotrophin-4, tropomyosin-related kinase b receptor, and p75 NTR receptor by high-resolution immunohistochemistry on the adult mouse neuromuscular junction. J Peripher Nerv Syst 15:40–49. https://doi.org/10.1111/j.1529-8027.2010.00250.x
101. Mantilla CB, Stowe JM, Sieck DC, Ermilov LG, Greising SM, Zhang C, Shokat KM, Sieck GC (2014) TrkB kinase activity maintains synaptic function and structural integrity at adult neuromuscular junctions. J Appl Physiol 117:910–920. https://doi.org/10.1152/japplphysiol.01386.2013
102. Sharma M, Burré J, Südhof TC (2011) CSPα promotes SNARE-complex assembly by chaperoning SNAP-25 during synaptic activity. Nat Cell Biol 13:30–39. https://doi.org/10.1038/ncb2131