COORDINATED EFFECTS OF SYNAPTIC ACTIVITY AND MUSCLE CONTRACTION ON CPKC REGULATION BY PDK1 AND BDNF/TRKB SIGNALLING

Erica Hurtado Caballero

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COORDINATED EFFECTS OF SYNAPTIC ACTIVITY AND MUSCLE CONTRACTION ON cPKC REGULATION BY PDK1 AND BDNF/TRKB SIGNALLING

An approach towards the amyotrophic lateral sclerosis pathophysiology

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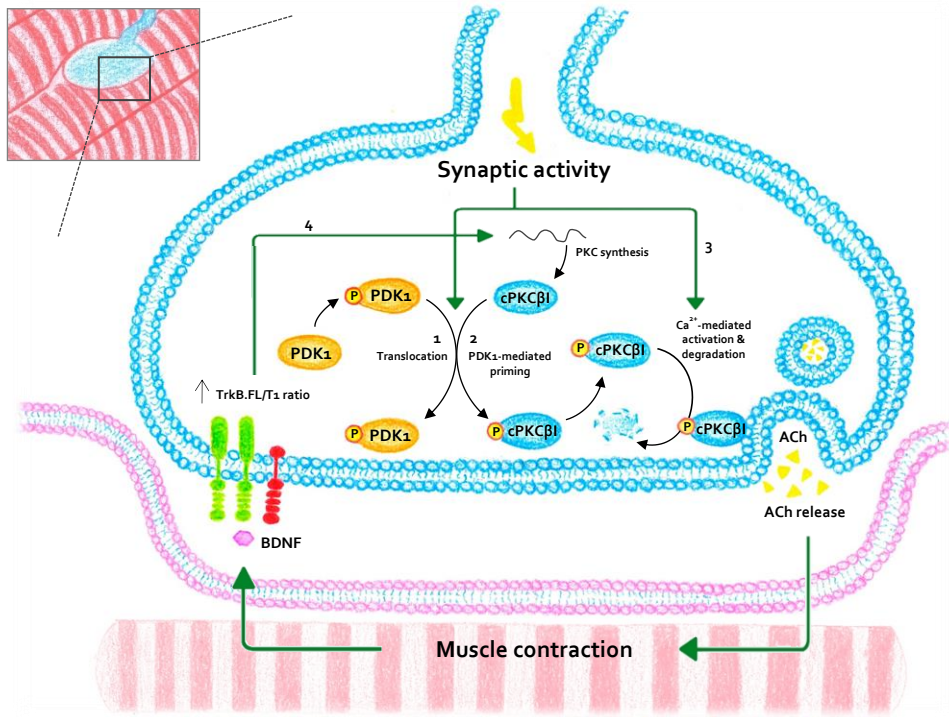
ERICA HURTADO CABALLERO



DOCTORAL THESIS 2017

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An approach towards the amyotrophic lateral sclerosis pathophysiology

DOCTORAL THESIS

Thesis supervisors:

Dr. María Ángel Lanuza

Dr. Neus Garcia

Prof. Josep Maria Tomàs

Unitat d'Histologia i Neurobiologia
Departament de Ciències Mèdiques Bàsiques
UNIVERSITAT ROVIRA I VIRGILI
Reus 2017

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UNIVERSITAT ROVIRA I VIRGILI
UNITAT D'HISTOLOGIA I NEUROBIOLOGIA
DEPARTAMENT DE CIÈNCIES MÈDIQUES BÀSIQUES
FACULTAT DE MEDICINA I CIÈNCIES DE LA SALUT

Maria Àngel Lanuza Escolano, professora titular del Departament de Ciències Mèdiques Bàsiques de la Universitat Rovira i Virgili.

Neus Garcia Sancho, professora titular del Departament de Ciències Mèdiques Bàsiques de la Universitat Rovira i Virgili.

Josep Maria Tomàs Ferré, catedràtic del Departament de Ciències Mèdiques Bàsiques de la Universitat Rovira i Virgili.

FAIG CONSTAR que aquest treball, titulat "COORDINATED EFFECTS OF SYNAPTIC ACTIVITY AND MUSCLE CONTRACTION ON cPKC REGULATION BY PDK1 AND BDNF/TRKB SIGNALLING. An approach towards the amyotrophic lateral sclerosis pathophysiology", que presenta Erica Hurtado Caballero per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Ciències Mèdiques Bàsiques d'aquesta Universitat i que acompleix els requeriments per poder optar a Menció Internacional.

Reus, 5 de setembre de 2017

La directora i els codirectors de la tesi doctoral,

Dr. Maria Àngel Lanuza Escolano Dr. Neus Garcia Sancho Prof. Josep Maria Tomàs Ferré

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"Every real story is a never ending story"

Michael Ende

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iii. ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
ADP	Adenosine diphosphate
Aloop	Activation-loop
ALS	Amyotrophic lateral sclerosis
aPKC	Atypical protein kinase C
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CaC	Calphostin C
CaM kinase	Ca^{2+} calmodulin-regulated protein kinase
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
cPKC	Conventional protein kinase C

DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EPC	Endplate current
EPP	End plate potential
FALS	Familial ALS
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
GDI Rho	GDP dissociation inhibitor
HGF	Hepatocyte growth factor
HM	Hydrophobic-motif
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycan
Hz	Herz
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IP_3	Inositol trisphosphate
KDa	Kilodalton
LAL	Levator auris longus

LIF	Leukemia inhibitory factor
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
mBDNF	Mature BDNF
mEPP	Miniature end plate potential
MHC	Myosin heavy chain
ml	Milliliter (10^{-3}l)
mM	Millimolar (10^{-3}M)
mV	Millivolts (10^{-3}V)
MN	Motoneuron
ms	Millisecond (10^{-3}s)
mTOR	Mammalian target of rapamycin
Na^+	Sodium ion
nAChR	nicotinic acetylcholine receptor
NF	Neurofilament
NGF	Nerve growth factor
NMJ	Neuromuscular junction
NT	Neurotrophin

NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
nPKC	Novel protein kinase C
$p75^{NTR}$	p75 neurotrophin receptor
PBS	Phosphate buffer saline
PDK1	3-Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PI (3,4) P2	Phosphatidylinositol (3, 4) bisphosphate
PI (4,5) P2	Phosphatidylinositol (4,5) bisphosphate
PI (3,4,5)P3	Phosphatidylinositol (3,4,5) trisphosphate
PKA	Protein kinase A
PLCγ	Phospholipase C gamma
PMA	Phorbol-12-myristate-13-acetate
PNS	Peripheral nervous system
PS	Phosphatidylserine
PSC	Perisynaptic Schwann cell
PTB	Phosphotyrosine binding domain containing proteins
PVDF	Polyvinylidene difluoride

qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RACK	Receptor for activated C-kinase
Rpm	Revolutions per minute
SD	Standard deviation
Ser	Serine
SEM	Standard Error of the Mean
SH2	Src homology 2
SOD1	Cu^{2+}/Zn^{2+} superoxide dismutase 1
SPSS	Statistical package for the social sciences
SR	sarcoplasmic reticulum
TBE	2,2,2 tribromoethanol
TBST	Tris-buffered saline-0.1% Tween-20
Thr	Threonine
TM	Turn-motif
TNF	Tumor necrosis factor
TRICT	Tetramethylrhodamine
TrkA	Tropomyosin-related kinase A receptor
TrkB	Tropomyosin-related kinase B receptor
TrkB.FL	Tropomyosin-related kinase B receptor full length

TrkB.T1	Tropomyosin-related kinase B receptor truncated isoform 1
TrkB.T2	Tropomyosin-related kinase B receptor truncated isoform 2
TrkC	Tropomyosin-related kinase C receptor
VDCC	Voltage-dependent calcium channel
α-BTX	α-bungarotoxin
βIV5-3	V5-containing cPKCßI inhibitor peptide
μ-CgTx-GIIIB	μ-conotoxin GIIIB
μg	Microgram (10^{-6}g)
μl	Microliter (10^{-6}l)
μm	Micrometer (10^{-6}m)
μM	Micromolar (10^{-6}M)

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ABSTRACT

The neuromuscular system is a complex and interconnected network in which presynaptic motoneurons and Schwann cells "tell" skeletal muscle to grow, to differentiate and how they should function. Conversely, skeletal muscle provides signals, including neurotrophins, that regulates the survival and function of motoneurons during development, maintenance and/or injury. Neurotrophins as BDNF, are regulated by activity and binding to TrkB trigger different pathways that impact on NMJ function, for example activating presynaptic PKCs. Thus, it is important to address how operates pre- and postsynaptic activities in physiological conditions to balance neuromuscular functionality through regulation of BDNF and PKC signalling. To address that, we stimulated the phrenic nerve of rat diaphragms with or without contraction to differentiate the effects of synaptic activity from that of muscle contraction. Then, we performed ELISA, Western Blot, qRT-PCR, immunofluorescence and electrophysiological techniques. Results showed that both synaptic activity and muscle contraction regulate cPKCβI maturation and activation in a complex and balanced way through PDK1 and BDNF/TrkB signalling. This regulation will determine NMJ functionality since our results also demonstrated that cPKCβI is directly involved in neurotransmission enhancing ACh release. However, what happens in a pathological context such us Amyotrophic lateral sclerosis (ALS) where neuromuscular activity is decreased? Could therapies as physical exercise, increasing activity, prevent the symptoms of ALS? To address that, we performed running and swimming-based training protocols to analyse the BDNF signalling in the plantaris muscle of SOD1-G93A mice by Western Blot. Results showed that in ALS disease where there is a loss of the connection between nerve and muscle, BDNF signalling is impaired but could be prevented in a different way depending on the nature and the intensity of the physical exercise imposed. Altogether, these results provide a mechanistic insight into the coordinated role of pre- and postsynaptic components to accurately preserve NMJ function.

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RESUMEN

El sistema neuromuscular es un complejo circuito interconectado en el cual las motoneuronas presinápticas y células de Schwann indican al músculo esquelético cómo crecer, diferenciarse y funcionar. Por otra parte, el músculo proporciona señales, incluyendo las neurotrofinas, que regulan la supervivencia y las funciones de las motoneuronas. Concretamente, la neurotrofina BDNF regulada por actividad, al unirse a TrkB, puede activar diferentes vías incluyendo las PKCs. Por consiguiente, es importante conocer cómo opera la actividad pre- y post-sináptica en condiciones fisiológicas para controlar la función neuromuscular a través de la regulación de BDNF y PKCs. Para realizarlo, se estimuló el nervio frénico del diafragma de rata bloqueando o no la contracción, para separar los efectos de la actividad sináptica y de la contracción muscular. A continuación, se realizaron las técnicas ELISA, Western Blot, qRT-PCR, inmunofluorescencia y electrofisiología. Los resultados mostraron que tanto la actividad sináptica como la contracción muscular regulan la maduración y la activación de la cPKCβI de una manera compleja y balanceada a través de PDK1 y BDNF/TrkB. Esto determinará la funcionalidad de la sinapsis porque los resultados también mostraron que cPKCβI potencia la liberación de acetilcolina. Sin embargo, ¿qué ocurre en la esclerosis lateral amiotrófica (ELA) donde la actividad neuromuscular disminuye? ¿Podría el ejercicio físico, incrementando la actividad neuromuscular, prevenir los síntomas? Para abordar esto, se realizaron protocolos de entrenamiento basados en la natación y el *running* y se analizó la vía del BDNF en el músculo plantaris de ratones SOD1-G93A mediante Western Blot. Los resultados mostraron que en ELA, la señalización del BDNF está alterada, pero se puede preservar este deterioro de diferente manera en función de la naturaleza y/o intensidad el ejercicio físico impuesto. En conjunto, aquí se muestra un planteamiento mecanicista del rol coordinado entre los componentes pre- y post-sinápticos para preservar la función sináptica.

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RESUM

El sistema neuromuscular és un complex circuit interconnectat en el qual les motoneurones presinàptiques i cèl·lules de Schwann indiquen al múscul esquelètic com créixer, diferenciar-se i funcionar. D'altra banda, el múscul proporciona senyals, incloent les neurotrofines, que regulen la supervivència i les funcions de les motoneurones. En concret, la neurotrofina BDNF regulada per activitat, a l'unir-se al TrkB, pot activar diferents vies incloent les PKCs. Per tant, és important conèixer com opera l'activitat pre- i post-sinàptica en condicions fisiològiques per controlar la funció neuromuscular a través de la regulació del BDNF i PKCs. Per estudiar això, es va estimular el nervi frènic del diafragma de rata amb o sense contracció, per tal de separar els efectes de l'activitat sinàptica i la contracció muscular. A continuació es van realitzar les tècniques ELISA, Western Blot, qRT-PCR, immunofluorescència i electrofisiologia. Els resultats van mostrar que tant l'activitat sinàptica com la contracció muscular regulen la maduració i l'activació de la cPKCβI d'una manera complexa i equilibrada a través de la PDK1 y del BDNF/TrkB. Això determinarà la funcionalitat de la sinapsi ja que els resultats també van mostrar que la cPKCβI potencia l'alliberament d'acetilcolina. No obstant, què ocorre en l'esclerosi lateral amiotròfica (ELA) on l'activitat neuromuscular disminueix? Podria l'exercici físic, incrementant l'activitat neuromuscular, prevenir els símptomes? Per abordar això, es van realitzar protocols d'entrenament basats en la natació i el *running* i es va analitzar la via del BDNF en el múscul plantaris de ratolins SOD1-G93A mitjançant Western Blot. Els resultats van mostrar que en l'ELA, la senyalització del BDNF està alterada però es pot prevenir aquesta afectació de manera diferent en funció de la natura i/o intensitat de l'exercici físic imposat. En conjunt, aquí es mostra un plantejament mecanicista del rol coordinat entre els components pre- i post-sinàptics per preservar la funció sinàptica.

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A. INTRODUCTION

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INTRODUCTION

The vertebrate neuromuscular system is a complex and coordinate linkage of muscles and nerves that includes:

- The skeletal muscles, which are generally attached to the skeletal system of bonds and joints, and produce the forces needed for movements and the maintenance of the body posture. The skeletal muscle is formed by fascicles of long contractile tubular cells called myocytes or muscle fibres.

- The motoneurons (MNs), which can be split into two main classes: upper and lower motor neurons. Upper MNs originate in the motor cortex in the brain and project their axons into the spinal cord. Lower MNs cell bodies lie in the ventral horn of the spinal cord, and are synapsed by upper MNs. The axons of lower MNs project into the periphery where they form synapses. There are two main classes of lower MNs: α-motor neurons and γ-motor neurons. α-motor neurons have large cell bodies and synapse extrafusal skeletal muscle, which is responsible for generating tension by contracting (Kanning et al., 2010). γ-motor neuron innervates intrafusal skeletal muscle that comprises the muscle spindle (a sensory organ involved in proprioception and monitoring muscle tension). γ-motor neurons are not responsible for eliciting muscle contraction (Kanning et al., 2010).

The specialised synaptic interface between α-motor neuron and the muscle fibre they innervate is known as the motor endplate (because of its platter-shaped outline) or neuromuscular junction (NMJ). This chemical synapse controls the excitation of skeletal muscle and its mechanical response, which results in physical movement.

Therefore, the neuromuscular system is comprised of individual motor units, each of which features a single α-motor neuron and all the muscle fibres it innervates (Baldwin et al., 2013). The MN and its muscle unit are inseparable in function

because each action potential in the neuron activates all fibres of the muscle unit. Thus, motor units are the indivisible quantal elements in all movements.

It is interesting to note that the nervous system and skeletal muscles are linked by two important mechanisms of control:

(1) Synaptic control, by which muscle contraction is initiated by nerve impulses generated in the brain cortex, the brainstem or through spinal cord neurons, and continue by depolarization of the sarcolemma and electromechanical coupling.

(2) Neurotrophic control, which depends on the release of soluble factors that regulate the development, differentiation, survival and function of neuromuscular system components (Cisterna et al., 2014). Within the neuromuscular system, the neurotrophic factors signalling at synapses involves MN and muscle fibres as well as perisynaptic glia (Funakoshi et al., 1993, 1995; Matthews et al., 2009; Pitts et al., 2006).

1. The neuromuscular junction

In the nervous system, information is propagated in networks of proper neuronal connections. A NMJ is the essential specialized unit for communication between a α-motor neuron, a target muscle cell, and surrounding Schwann cells. The position of NMJs on the muscle fibre, the configuration of the nerve terminals within the NMJ, and the extent of development of the postsynaptic region can vary according to phylum and species, between different muscles in a given species, and between different fibres in a given muscle. Despite these differences, all NMJs have four principal components (Figure 1): 1. A Schwann cell that forms a cap above that portion of the nerve terminal that does not face the postsynaptic region. 2. A nerve terminal that contains the neurotransmitter. 3. A synaptic cleft lined with a basal lamina. 4. A postsynaptic membrane that contains the receptors for the neurotransmitter. In vertebrate voluntary muscle, the neurotransmitter is

acetylcholine (ACh), the receptor is the nicotinic acetylcholine receptor (nAChR), and the synaptic space contains acetylcholine esterase (AChE).

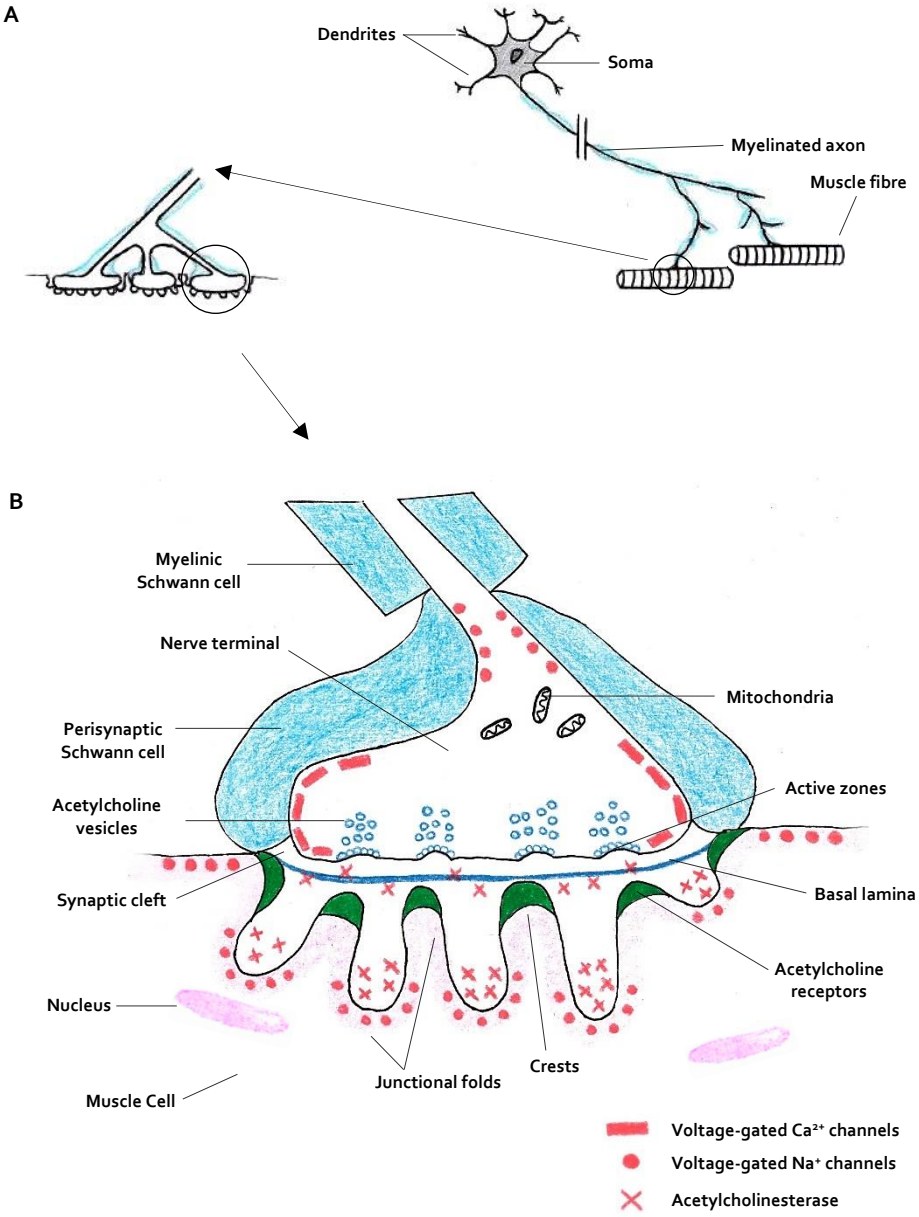

Figure 1. Motor neuron and neuromuscular junction structure. (A) Motor neuron, making synaptic contact with several muscle fibres, together forming the motor unit. **(B)** In the NMJ, the nerve terminal is positioned in the primary folds of the muscle fibre membrane and covered with the perisynaptic Schwann cells (in blue). Nerve terminal contains synaptic vesicles filled with ACh and upon arrival of a presynaptic action potential the vesicles fuse with the presynaptic membrane at the active zones (releasing ACh in the synaptic cleft). AChRs (in dark green) are located in the crests of the junctional folds and bind ACh, triggering an ion current flow which underlies a depolarization that activates voltage gated Na+ channels located in the depths of the folds. This results in a muscle action potential that eventually induces contraction of the fibre. Adapted from Physiology of the Neuromuscular Junction, Dr Clare Ackroyd and Dr C Gwinnutt by P. Cerezuela.

1.1. Schwann cells

In the Peripheral Nervous System (PNS), every neuron axon is wrapped by highly specialised cells termed Schwann cells, which provide them with both structural and metabolic support. Within a peripheral nerve, there are both myelinated and non-myelinated axons, but all PNS axons interact with Schwann cells (Stevens and Lowe, 2005). In a NMJ, the perisynaptic Schwann cell (PSCs; also known as terminal Schwann cells), which wrap around the nerve terminal, contacts with the myocyte and encloses the synaptic cleft, is amyelinic. On the other hand, the immediately above Schwann cell wraps the axon in the typical myelinogenic manner (Figue 1; Young et al., 2014).

PSCs have functional L-type voltage dependent calcium channels (VDCC) (Robitaille et al., 1996), muscarinic acetylcholine receptors (mAChR) (Georgiou and Charlton, 1999; Jahromi et al., 1992; Robitaille et al., 1997), purinergic (Robitaille, 1995), and substance P (SP) receptors (Robitaille et al., 1996). PSCs express receptors for neurotransmitters that have been identified at the NMJ (Ko and Robitaille, 2015), such as ACh and adenosine triphosphate (ATP). Moreover, the neurotrophins (NTs) and their receptors, are also found on PSCs (Belluardo et al., 2001; Funakoshi et al., 1993; Koliatsos et al., 1993; Loeb et al., 2002). NT availability at the NMJ is related to synapse activity, which is in agreement with the robust changes in synapse structure associated with different activity levels. The anatomical relationship between PSCs and the presynaptic and postsynaptic

elements of the NMJ, as well as the presence of receptors capable of detecting neurotransmission or substances able to modulate neurotransmission suggest that synaptic activity is likely to provoke PSC responses that could modulate subsequent synapse activity (Ko and Robitaille, 2015; Lin and Bennett, 2006; Rochon et al., 2001; Todd et al., 2007, 2010).

1.2. Presynaptic region: the nerve terminal

Inside the target muscle (and, to some extent, in the nerve trunk) each motor axon splits into many terminal branches. Normally, each adult twitch muscle fibre receives only one terminal branch called nerve terminal, typically located in the middle between the two ends of the fibre. In mammals, the presynaptic nerve terminal splits up into several small twigs when contacting a muscle fibre called synaptic end boutons. These final branches are thin (about 2µm), unmyelinated, and covered with Schwann cells but retains a rich concentration of membrane associated voltage gated ion channels (Figure 1). The presynaptic nerve ending is densely packed with small vesicles (synaptic vesicles) about 50-60 nm in diameter, containing the neurotransmitter ACh. They also contain other compounds, such as ATP, which are co-released with ACh and are thought to play a particularly important signalling role during development. These vesicles are not present in a random array; there are electron-dense guiding structures which leads vesicles towards membrane sites, referred as active zones, at which their contents can be released by exocytosis. In particular, active zones contain high levels of VDCC and proteins involved in the fusion of synaptic vesicles with the terminal plasma membrane (Zhai and Bellen, 2004). A queue of vesicles waits in front of each release site, and these sites tend to be opposite to postsynaptic secondary clefts. ACh is synthetized by choline acetyltransferase (ChAT), a transferase enzyme that is produced in the body of the neuron and is transported to the nerve terminal, where its concentration is highest. Both ACh production and loading of ACh into synaptic vesicles depend (indirectly) on active transport processes that require energy. To provide this energy, motor nerve terminals contain large numbers of

mitochondria, located near the upper regions of the terminal, away from the muscle-facing surface. In addition to the clear synaptic vesicles, a small number of larger (70-100 nm) dense-core vesicles are also present. Although their function is unknown; they may contain neurotrophic substances with an influence on muscle (e.g. Calcitonin gene-related peptide; CGRP). Moreover, presynaptic terminal contains neurotubuli, mAChR which have functions in the maintenance and synaptic efficacy (Abbs and Joseph, 1981; Caulfield, 1993; Ganguly and Das, 1979; Nathanson, 2000; Parnas et al., 2000; Santafé et al., 2006), purinergic receptors (Garcia et al., 2013) and NT receptors such as tropomyosin-related kinase B receptor (TrkB) (Garcia et al., 2010a; Gonzalez et al., 1999).

1.3. Synaptic cleft lined with a basal lamina

A synaptic cleft separates the pre- and post-synaptic components of the NMJ, spanning ~70 nm with the basal lamina, a highly specialized extracellular matrix (ECM) made up of several molecules (Figure 1). AChE, the enzyme responsible for the degradation of ACh, is found in high concentrations in these areas (Rotundo, 2003). Concretely, the catalytic subunits of AChE are anchored into the basal lamina of the postsynaptic membrane by a collagen-like tail (Ohno et al., 2000). Some experimental studies of muscle innervation *in vitro* indicate that both cells contribute to AChE synthesis; most of the AChE comes from the muscle and a smaller fraction from the MN (Jevsek et al., 2004). AChE activity is inhibited by high concentrations of its substrate, ACh. Thus, only a small amount of ACh is metabolized before it reaches the postsynaptic receptors. The synaptic basal lamina plays an important role in NMJ development and regeneration, and in specifying the molecular architecture and physiologic properties of the pre- and postsynaptic membranes. Thus, the synaptic basal lamina contains factors that guide regenerating nerve terminals to previously denervated NMJs, induce physiologic and morphologic maturation of the nerve terminal even in the absence of the muscle fibre. Also, induce regeneration of the junctional folds and insertion of AChR into the folds even in the absence of the nerve terminal (Burden et al.,

1979; Glicksman and Sanes, 1983; Hall and Sanes, 1993; Sanes, 2003). The basal lamina (10-15 nm thick) completely surrounds the external membrane (plasmalemma) of the whole muscle fibre, extends throughout the central portions of the primary and secondary synaptic clefts. Is comprised of arrays of laminin, collagen, heparan sulfate proteoglycan (HSPG) to facilitate cell adhesion and signalling processes. The short distance of the synaptic cleft in combination with the high concentrations of neurotransmitter promote the rapid diffusion of ACh to the opposite post-synaptic muscle membrane. Finally, AChE degrades ACh into 4 acetate and choline to terminate the signal efficiently and to halt the nerve impulse (Rotundo, 2003).

1.4. Postsynaptic region: the muscle fibre

The skeletal muscle is formed by fascicles of long contractile tubular cells called myocytes or muscle fibres. It is a cylindrical and multinucleated cell with a highly-organized structure that is formed by the fusion of myoblast cells during development. The resultant cell membrane is called sarcolemma and their fused cytoplasm is called sarcoplasm.

The postsynaptic region of the muscle fibre consists of junctional folds in the postsynaptic membrane and junctional sarcoplasm of the muscle fibre (Figure 1). The postsynaptic region of the muscle fibre is specialized to respond efficiently and rapidly to the neurotransmitter from the overlying nerve terminal. The post-synaptic membrane is enriched with AChRs opposite the active zone, the density of which (>10,000 receptors/μm2) is extremely high compared to the non-synaptic area (~10 receptors/μm2 in adults). The post-synaptic membrane invaginates to form junctional folds beneath the nerve terminal. AChRs are concentrated at the crests and upper parts of these folds. This organization ensures that acetylcholine encounters a high concentration of AChRs within microseconds of release by the nerve terminal, thereby facilitating membrane depolarization and favouring efficient neuromuscular function. On the vertebrate skeletal muscle fibre, ACh

binds to the AChRs to open the ligand-gated sodium channels, allowing sodium influx to initiate muscle contraction.

Continuously with the base of the junctional folds, it is found the junctional sarcoplasm. The amount of junctional sarcoplasm differs between different NMJs, and even between different regions at a given NMJ. The junctional sarcoplasm contains a varying complement of mitochondria, smooth and rough endoplasmic reticulum, Golgi cisternae, lysosomal structures, small clear vesicles, microtubules, intermediate filaments, and scattered glycogen granules. The region is traversed by transverse tubules (T-tubules) that open into the secondary synaptic clefts. T-tubules contact with sarcoplasmic reticulum (SR) and transmit the stimuli coming from the neuron inside the myocyte. The known metabolic functions of the junctional sarcoplasm include the synthesis and degradation of AChR, synthesis of the end-plate specific species of AChE, and regulation of the subsynaptic ionic environment. Multiple nuclei are adjacent to, or intermingle with, the junctional sarcoplasm at each NMJ. At the mature NMJ, subsynaptic nuclei are specialized to selectively transcribe mRNA for AChR subunits and for other NMJ-specific proteins (Klarsfeld et al., 1991; Sanes et al., 1991; Simon et al., 1992).

2. Synaptic control

In all vertebrates, neuromuscular transmission involves the release of ACh from the nerve terminal and its action on the muscle to trigger its excitation and contraction. The NMJ is highly specialized to ensure that the main events in this complex process take place within less than a millisecond.

2.1. From action potential to muscle contraction

As excitable cells, neurons can alter transitorily their membrane potential to transmit electrical signals along their axons. This activity is achieved by significant

influx or efflux of ions, thus modifying the typical charge gradient between intra- and extracellular compartments.

When the motor neuron integrates the thousands of synaptic inputs that receives, it sends an action potential to the distal nerve terminal. Within the cytoplasm of the presynaptic nerve terminal ACh is contained in membrane-bound vesicles. Each vesicle is believed to contain about 10.000 ACh molecules, which can be released within 100 ms or so following a nerve impulse. These multi-molecular packets are the elementary units of evoked ACh release. They are often referred to as "quanta" of ACh, by analogy with the elementary units of electromagnetic energy, and their release as "quantal" release. The action potential induces the aperture of the VDCC, present on the presynaptic membrane, thereby causing an increase in calcium influx which results in a significant increase in the rate of vesicle fusion and exocytosis (Engel, 2008; Hughes et al., 2006; Robitaille et al., 1990; Wood and Slater, 2001).

The mechanism for ACh release from the nerve is complemented by a high density of AChRs in the postsynaptic membrane of the muscle fibre. These ligand-gated ion channels open briefly when ACh binds to them. This causes an influx of positive ions and a transient local depolarization of the muscle membrane. Since the NMJ is sometimes referred to as the endplate, the signals associated with this charge influx and subsequent depolarization are known as the endplate current (EPC), and the endplate potential (EPP), respectively. The analogous much smaller signals that result from the impact of spontaneously released single quanta of ACh are known as miniature EPCs and EPPs. The EPP typically rises to a peak of some 25–45 mV (from the resting potential of about 75 mV) in about 1 ms. As it does so, it causes the opening of a second class of ion channels in the muscle fibre, the voltage-gated Na^+ channels, thus initiating an action potential in the muscle fibre. The action of the ACh is rapidly terminated by the action of the AChE associated with the synaptic basal lamina, splitting ACh into acetate and choline. The choline is subsequently transported by a high affinity uptake system into the nerve terminal where it is eventually used to make more ACh.

Muscle contraction

The muscle fibre is broken down into myofibrils and there are many myofibril units in each muscle fibre or cell (Figure 2). Each myofibril consists of a series of sarcomeres which are the smallest units of contraction (Clark et al., 2002). The sarcomeres consist of highly organized protein assemblies that give the muscle fibre a characteristic striated appearance. Contains longitudinal arrays of thick and thin filaments (myofilaments) that are maintained in a hexagonal lattice by a scaffolding network. The thin filaments consist of filamentous actin entwined by tropomyosin and troponin, a calcium-binding protein. Thick filaments consist of myosin, a large molecule with heavy and light chains. The myosin heavy chains have a tail region and a globular head that can bind to the active sites on actin. Myosin heads have ATPase activity, which is essential for the muscular contraction process. Its ability to break up ATP to adenosine diphosphate (ADP) will allow it to bind actin filaments and slide them. Thick filaments are formed by the assembly of myosin monomers with their tails centrally and heads protruding outwards, with an antiparallel orientation on opposite ends of the filament. The part dark stripe in the middle of each sarcomere containing the thick myosin filaments with some overlapping of the "loose" ends of the thinner actin filaments is called the A band. The lighter I band contains only actin filaments. It is the alternating pattern of these A and I bands that gives skeletal muscle fibres their typical striated appearance. The H bands represent the distance between the ends of the actin fibres within the A band. The Z line is found at the other end of the actin fibres and mark the border between two different sarcomeres (Figure 2; Lange et al., 2006).

When contraction is initiated by a muscle fibre action potential, calcium is released from the SR binds troponin, uncovering binding sites on actin. This leads to the formation of cross-bridges between actin and myosin. The ATPase activity of myosin is enhanced by formation of cross-bridges, and when ATP is hydrolysed the cross bridge is broken, freeing the myosin head to swivel to the next actin-binding site. The repeated formation and cleavage of actomyosin cross-bridges produces

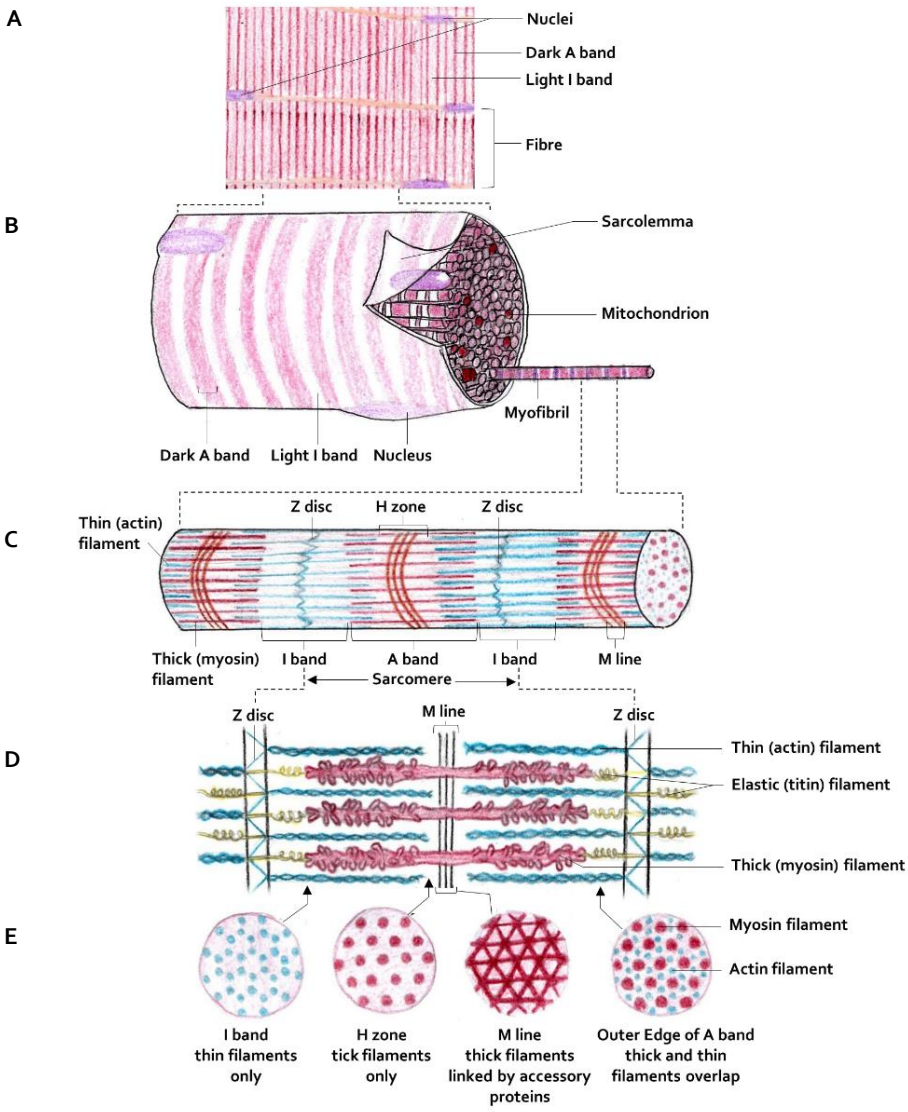

Figure 2. Microscopic anatomy of a skeletal muscle fibre. (A) Photomicrograph of portions of two isolated muscle fibres (700x). Notice the striations (alternating dark and light bands). **(B)** Diagram of part of a muscle fibre showing the myofibrils. One myofibril extends from the cut end of the fibre. **(C)** Small part of one myofibril enlarged to show the myofilaments responsible for the banding pattern. Each sarcomere extends from one Z disc to the next. **(D)** Enlargement of one sarcomere (sectioned lengthwise). Notice the myosin heads on the thick filaments. **(E)** Cross-sectional view of a sarcomere cut through in different locations. Adapted from Pearson Education by P. Cerezuela.

the sliding action of thin and thick filaments that causes shortening of the sarcomere and muscle contraction (Cooke, 2004; Huxley, 2000). The actomyosin cross-bridges serve as the mechanical linkage between thick and thin filaments for transmitting tension to the insertions of the muscle fibre. The amount of tension is proportional to the number of cross-bridges, reaching a maximum at sarcomere lengths when thick and thin filaments have the greatest overlap (Gordon et al., 1966; Hill and White, 1968).

Histological and physiological studies have shown that most muscles contain a mixture of muscle fibres with differing contraction speeds and force outputs. The isoform of the myosin heavy chain (MHC) expressed in the muscle fibre is one of the most important factors influencing the speed of contraction, because the rate of ATP hydrolysis determines the speed of cross-bridge cycling and sarcomere shortening (Bottinelli et al., 1996; Harridge et al., 1996). There are four major isoforms of MHC expressed in mammalian skeletal muscles: MHC I, also called slow myosin; and the three fast isoforms, MHC IIA and MHC IIX (also called MHCIID) and MHCIIB. Type I muscle fibres have a slow twitch and use oxidative metabolism. Type I fibres express MHC I, the slow isoform of myosin, and contain many mitochondria. The metabolic profile and vascularization render Type I muscle fibres highly resistant to fatigue, and thus suitable for sustained contraction under aerobic conditions. Type II muscle fibres are fast-twitch fibres, expressing fast isoforms of myosin which exhibit strong ATPase activity at alkaline pH. Type IIA fibres express the MHC IIA isoform of myosin and mitochondria are relatively abundant. In addition, Type IIA fibres contain glycolytic enzymes, such as phosphorylase, and have abundant glycogen stores. These metabolic properties allow Type IIA to function under aerobic and anaerobic conditions, and provide them with a fairly high resistance to fatigue. Type IIX fibres express the fast isoform of myosin, MHC IIX (also known as IID). Type IIX fibres have relatively sparse mitochondria, but contain glycolytic enzymes and stores of glycogen. Type IIX muscle fibres fatigue easily, but are suitable for short bursts of anaerobic exercise. Type IIB fibres express a very fast form of myosin, the MHC IIB isoform, particularly

in muscles with very fast speeds of contraction (Acevedo and Rivero, 2006; Rivero et al., 1997, 1998; Rome, 2006). They produce ATP at a slow rate by anaerobic metabolism and break it down very quickly. This results in short, fast bursts of power and rapid fatigue.

Mammalian skeletal muscles are heterogeneous in nature and we can also find the coexpression of different MHC isoforms in a fibre resulting in the formation of hybrid fibres, which can be subdivided based on the predominant MHC isoform.

3. Neurotrophic control

Studies from mammalian NMJ have suggested that retrograde factors from muscle fibres are vital for the stability of motor nerve terminals. Segments of nerve terminal branches retracted rapidly after blockade of AChRs or removal of muscle fibres (Balice-Gordon and Lichtman, 1994; Rich and Lichtman, 1989). Neurotrophic control is responsible for the growth and survival of neurons and maintenance of mature neurons and is mediated by the secretion of diffusible molecules in the neuromuscular junction (Barde, 1990). Since the discovery of nerve growth factor (NGF) by Levi-Montalcini, more than a dozen neurotrophic molecules have been characterized (Levi-Montalcini, 1987; Poo, 2001; Sendtner et al., 2000). The group of neurotrophic factors comprises the family of neurotrophins (NTs), the ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) family, the hepatocyte growth factor (HGF) family, the family of insulin-like growth factors (IGFs), as well as the family of glial-derived neurotrophic factors (GDNF) (Sendtner et al., 2000). Among these neurotrophic factor families, the best studied group is NTs that comprise small and closely related proteins (Bibel and Barde, 2000).

NTs can be broadly understood as any secreted factor that has nourishing, sustaining and surviving effect on neurons and they are critical for the development and maintenance of the nervous system (Skaper, 2012). They have emerged as a key regulator of synaptic plasticity, supporting neuronal connectivity and

protecting neurons from cell death in both, Central Nervous System (CNS) and PNS (Chao et al., 2006; Kalb, 2005; Lewin and Barde, 1996; Oppenheim, 1991; Reichardt, 2006; Riffault et al., 2014; Ruit et al., 1990). However, accumulating evidence suggests that NTs play a more widespread role than originally thought. Accordingly, they are the focus of study in numerous cell populations across multiple tissue systems. Concretely, skeletal muscle acts as an abundant source of neurotrophic support throughout development and expresses several NT receptors (Gonzalez et al., 1999; Griesbeck et al., 1995; Ip et al., 2001; Kablar and Belliveau, 2005; Pitts et al., 2006; Sheard et al., 2002; Tessarollo et al., 1993). In adulthood, there is well established a neurotrophic control interdependence between glial cells and motor neurons (Ko and Robitaille, 2015; Michailov et al., 2004; Schulz et al., 2014). However, more knowledge it is necessary to understand the relationship between neurons, NTs and trophic actions on myofibres *in vivo*.

Currently the mammalian NT family consists of four structurally and functionally similar members: Nerve Growth Factor (NGF), Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5), which share 50 % similar amino acid sequence. They are dimeric ligands initially synthesized as precursors or pro-neurotrophins, which are cleaved to produce the mature proteins (Mowla et al., 2001). The actions of NTs depend on two different transmembrane-receptor signalling systems: the Tropomyosin related kinase (Trk) receptor and p75 neurotrophin receptor ($p75^{NTR}$) (Chao and Hempstead, 1995). NTs and their receptors are expressed by all the cellular components of the neuromuscular system (Funakoshi et al., 1993; Garcia et al., 2010b). Each proneurotrophin binds $p75^{NTR}$ which belongs to the Tumor Necrosis Factor (TNF) super family of receptors and signals cell apoptosis (Lewin and Barde, 1996; McAllister et al., 1999).

Following maturation through proteolysis, each mature NT bind with low affinity $p75^{NTR}$ ($Kd \approx 10^{-9}$ M) and the beneficial effects of NTs binding occur due to their selective docking to the Trk receptors with high affinity ($Kd \approx 10^{-11}$ M) (Dechant and Barde, 2002; Kaplan and Miller, 2000).

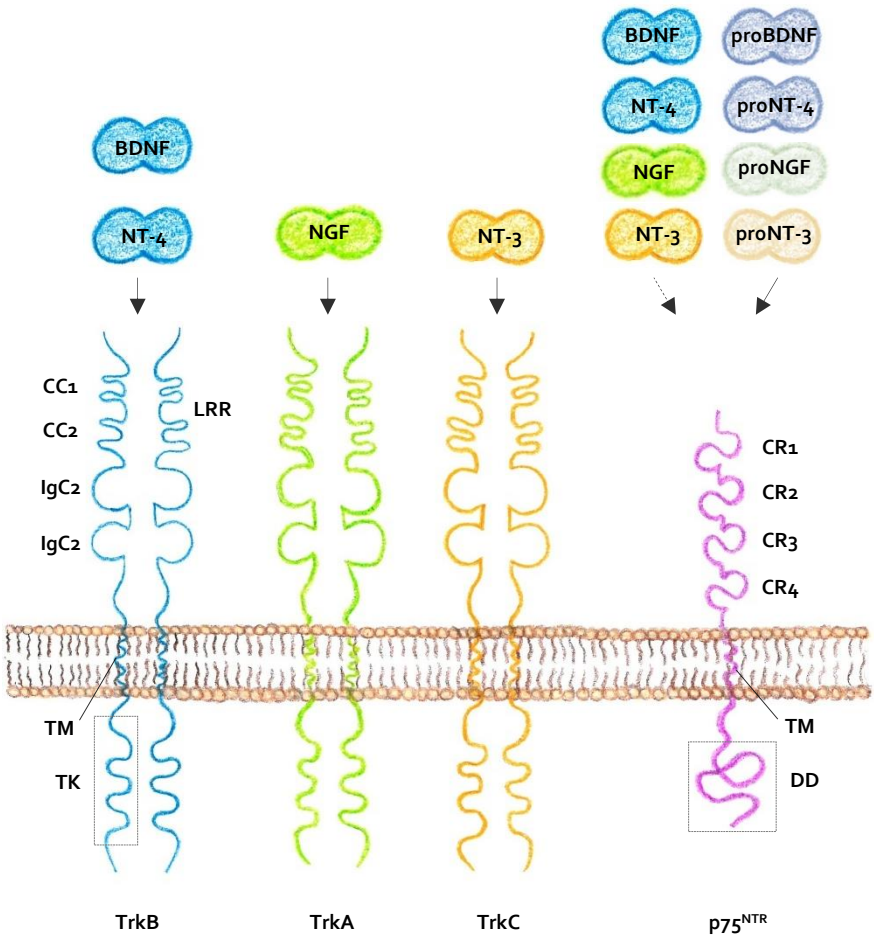

Figure 3. Neurotrophin–receptor interaction. Each proneurotrophin binds $p75^{NTR}$ and following its maturation, each mature NT is able to bind and activate $p75^{NTR}$, but with more specificity to Trk receptors. NGF recognize specifically TrkA; BDNF and NT_4 bind TrkB; NT_3 activates TrkC. In some cellular contexts, NT_3 is also able to activate TrkA and TrkB with less efficiency. Trk receptors are transmembrane (TM) proteins with a tyrosine kinase (TK) domain and an extracellular region. The extracellular region is composed by two cysteine-rich motifs (CC_1 and CC_2), a leucine-rich repeats (LRR) and two immunoglobulin-like domains (IgC_2). $P75^{NTR}$ has an extracellular domain that includes four cysteine-rich motifs (CR_1-CR_4), a single transmembrane domain and a cytoplasmic domain that includes a 'death' domain (DD). Adapted from Reichardt, 2006 by P. Cerezuela.

The function of these multiple receptors is complex since $p75^{NTR}$ and Trk receptors can function independently, but in neurons that express both $p75^{NTR}$ and Trk, they interact physically and functionally in ways that may alter the signalling properties

of each (Bibel and Barde, 2000; Dechant and Barde, 2002; Roux and Barker, 2002). Even though they are selective - NGF binds preferentially to tropomyosin-related kinase receptor A (TrkA); BDNF and NT4/5 to TrkB; and neurotrophin 3 (NT3) to tropomyosin-related kinase receptor C (TrkC) - they are not specific as for example NT-3 can bind to TrkA (but with lower affinity than to TrkC) (Figure 3; Pattarawarapan and Burgess, 2003). NT signalling through Trk receptors regulates cell survival, proliferation, the fate of neural precursors, axon and dendrite growth and patterning, and the expression and activity of functionally important proteins, such as ion channels and neurotransmitter receptors (Huang and Reichardt, 2003).

In addition, each proneurotrophin preferentially binds $p75^{NTR}$, rather than the Trk receptors. For decades, proneurotrophins were thought to be biologically inactive; the dogma was changed when in 2001, Hempstead and colleagues showed that proneurotrophins binding to $p75^{NTR}$ promote cell death (Beattie et al., 2002; Hempstead, 2002; Lee et al., 2001). Thus, the interaction of mature NTs with Trk receptors preferentially leads to cell survival, whereas binding of proneurotrophins to $p75^{NTR}$ leads to apoptosis (Beattie et al., 2002; Harrington et al., 2004; Hempstead, 2002; Lee et al., 2001; Teng et al., 2005, 2010; Volosin et al., 2006).

This thesis is focused on BDNF signalling pathway that leads to protein kinase C (PKC) activation. Therefore, following an overview of neurotrophic factors, BDNF and its receptors will be further developed and discussed.

3.1 Brain-derived neurotrophic factor

BDNF was first purified from the mammalian brain based on its survival-promoting action on dorsal root ganglion cells (Barde et al., 1982), and was classified as the second member of the NT family of growth factors, after NGF (Cohen et al., 1954).

Gene structure and regulation

The structural organization of the *bdnf* gene has been reviewed for mouse and rat (Aid et al., 2007; Liu et al., 2006), and for human (Liu et al., 2005). Its structure and regulation is complex: the *bdnf* gene produces 11 primary transcripts in rodents and 17 in humans, and each is characterized by a different 5-untranslated (UTR) exon linked by alternative splicing to a common exon encoding the protein and the 3UTR (Figure 4). Because the 3UTR contains two polyadenylation sites, each primary transcript can exist in two forms, one with a short and the other with a long 3UTR, producing a total of 22 (in rodents) or 34 (in humans) possible transcripts (Aid et al., 2007; Pruunsild et al., 2007). All of transcripts are translated into an identical mature dimeric protein, suggesting a multilevel regulation of expression (Aid et al., 2007).

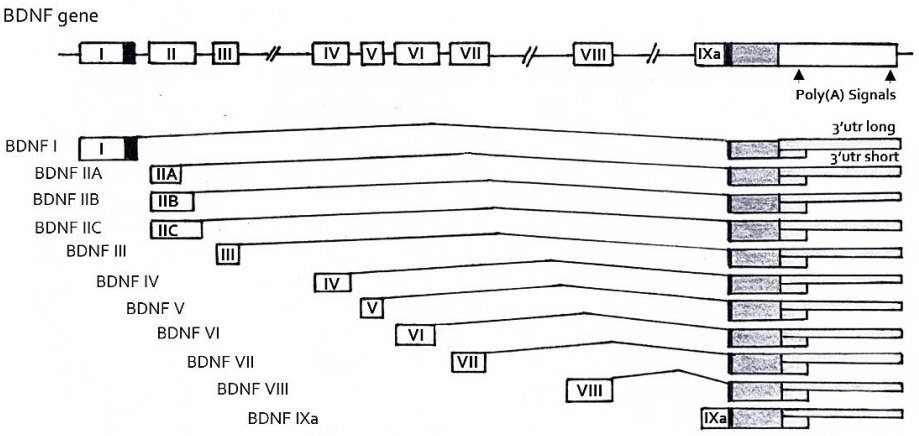

Figure 4. Mouse and rat *bdnf* gene structure and transcripts. Schematic representation of *bdnf* gene and its alternative transcripts. Adapted from Aid et al., 2007.

Bdnf transcripts are widely distributed, concretely, mouse and rat *bdnf* novel exons III, V, VII, VIII, and IXA are differentially expressed in adult brain and in peripheral tissues. In general, exons I, II, and III have brain-enriched expression patterns and exons IV, V, and VI are widely expressed also in nonneural tissues (Aid et al., 2007).

In addition, several studies show that the different BDNF mRNA isoforms are localized to different subcellular areas (Calabrese et al., 2007; Chiaruttini et al., 2008; Pattabiraman et al., 2005; Tongiorgi et al., 1997, 2004).

The complexity of the *bdnf* gene allows precise temporal and spatial regulation of BDNF expression. The exons are controlled by distinct promoters that are regulated by many stimuli depending on tissue-specificity and neural activity, age as well as hormone exposure (Koibuchi et al., 1999; Lauterborn et al., 1996; Oliff et al., 1998; Russo-Neustadt et al., 2000). These stimuli can activate different transcription factors, which can then bind to different promoter regions of the *bdnf* exons resulting in transcription of specific bdnf transcripts (West et al., 2014).

The best studied and probably the most potent BDNF transcription-inducing stimulus is neuronal activity. It has been established that *bdnf* gene is strongly regulated by neural activity through calcium-mediated pathways (Aid et al., 2007; Mellstrom et al., 2004; Shieh and Ghosh, 1999; West et al., 2001). Specifically, the expression of *bdnf* exons I, II and IV is regulated in an activity-dependent manner (West et al., 2014) and *bdnf* exon IV expression is strongly induced by elevations in intracellular calcium concentration (Hong et al., 2008; Tao et al., 1998). Moreover, kainic acid treatment induces a rise in intracellular Ca^{2+} levels and differential activation of V, VII, VIII, and IXA BNDF mRNAs promoters in the hippocampus and cerebral cortex of adult rat brain (Aid et al., 2007; Timmusk et al., 1993). In addition, increased neuronal activity following physical exercise increase bdnf transcription (Chen and Russo-Neustadt, 2009; Neeper et al., 1996; Russo-Neustadt et al., 2000). The significance of this activity-regulated transcription of *bdnf* is emphasized by the fact that BDNF is one of the major regulators of neuronal activity-dependent neurotransmission and plasticity (Bramham and Messaoudi, 2005; Lu, 2003; Poo, 2001; Schinder and Poo, 2000).

Processing, trafficking and secretion

Like all NTs, BDNF mRNA is translated into a precursor protein and then cleaved to yield the mature form of the protein which consists of 118- 120 amino acids. BDNF is synthesized as a pre-proBDNF, which enters into the endoplasmic reticulum (ER) lumen through its N-terminal 'pre' sequence (signal peptide) (Greenberg et al., 2009). The pre-sequence is removed by signal peptidases in the rough ER, resulting in a 32-kDa proBDNF protein. The proBDNF isoform is then N-glycosylated and glycosulfated (Mowla et al., 2001). The glycosylation increases the stability of proBDNF during processing and subcellular trafficking. The pro-domain participates in the proper folding and intracellular sorting of BDNF (Brigadski et al., 2005; Lee et al., 2001) and is not an inactive precursor of BDNF, but rather it is a signalling protein in its own right (Pang et al., 2004). ProBDNF isoform can be cleaved to produce the 14 kDa mature BDNF (mBDNF) inside the cell in trans-Golgi network or post-Golgi compartments by pro-convertases and furin. Moreover, proBDNF isoform can also be secreted as proBDNF without subsequent cleavage or then cleaved outside the cell by matrix metalloproteinases (MMP-7 or MMP-9) or plasmin (Lee et al., 2001; Lessmann et al., 2003; Pang et al., 2004; Seidah et al., 1996). The mature proteins, which are about 14 kDa in size are form stable and non-covalent dimers.

Nevertheless, both proBDNF and mBDNF are preferentially sorted and packaged into secretory vesicles. BDNF can be secreted to the extracellular space in a constitutive (i.e., spontaneous release) or regulated (i.e., in response to neural activity) manner. This secretion is different between neuronal and non-neuronal cells; Non-neuronal cell types (i.e., smooth muscle cells, fibroblasts) typically have only the constitutive secretory pathway since they may not express molecular components of the regulated secretory pathway (Kelly, 1985). However, neurons and neuroendocrine cells have distinct regulated and constitutive secretory pathways, although BDNF selectively traffics through the regulated, rather than the constitutive, secretory pathway (related to its activity-dependent regulation) (Farhadi et al., 2000; Griesbeck et al., 1999; Hibbert et al., 2003; Mowla et al., 1999).

There are no studies reporting how NTs are sorted to either constitutive or regulated pathways, but it is known that a polymorphism in the prodomain region (BDNF val to met) reduces the activity-dependent secretion of BDNF through interaction with sortilin (Bronfman et al., 2014; Chen et al., 2004, 2005).

It has been shown that NTs can be secreted not only by target tissues, which can be postsynaptic neurons or other types of cells, such as muscle, but also by presynaptic neurons, astrocytes, microglia, and glial cells, such as Schwann cells and oligodendrocytes, having paracrine and autocrine actions on neurons and other cell types (Bagayogo and Dreyfus, 2009; Bessis et al., 2007; Cao and Ko, 2007; Dai et al., 2001; Lessmann et al., 2003; Ohta et al., 2010; Schinder and Poo, 2000; Verderio et al., 2007; Yune et al., 2007). In the NMJ, the neurotrophic control between Schwann cell, pre- and postsynaptic sites is crucial for the maintenance of neuromuscular transmission (Gómez-Pinilla, 2011; Gómez-Pinilla et al., 2002; Huang and Reichardt, 2001; Jiang et al., 2008; Lipsky and Marini, 2007; Mantilla and Ermilov, 2012; Peng et al., 2003; Seeburger and Springer, 1993; Springer et al., 1995).

3.2 Tropomyosin related kinase B (TrkB)

After secretion, BDNF binds its receptors (TrkB or $p75^{NTR}$), which can be located along the axon, in the neuronal cell body, or at the NMJ (presynaptic neuron, postsynaptic muscle cell and Schwann cell).

TrkB receptor was first cloned from mouse brain tissue because of its high sequence homology to the NGF receptor TrkA (Klein et al., 1989). It is a high affinity receptor for BDNF and NT- 4/5 and, in a lower affinity, NT-3.

Gene and structure

The *TrkB* gene (NTRK2) is located on chromosome 9q22, consists of 24 exons and can produce multiple transcripts (Nakagawara et al., 1995; Stoilov et al., 2002; Valent et al., 1997). *TrkB* exons can be grouped into six clusters (Figure 5); the first

four exons contain G/C rich internal initiation entry sites (IRES) and recruit transcription factors to transcription start sites (which are located in exon 5) (Dobson et al., 2005; Stoilov et al., 2002). The four most abundant gene products are derived from translation start codon and alternative splicing: the full-length TrkB (TrkB.FL; gp145), the C-terminal truncated receptor 1 (TrkB.T1; gp95), a C-terminal truncated Shc+ receptor (T-shc in humans; T2 in rat). And finally, the TrkB-T-TK, a C-terminal truncated receptor lacking exon 23 and 24 but retaining tyrosine kinase activity (Luberg et al., 2010). Exons 5–14 encode the extracellular domain (ECD) of the TrkB receptor which contains a signal sequence for membrane localization, post-translationally glycosylated cysteine and leucine rich regions, and two immunoglobulin-like (IG-like) domains (Fenner, 2012; Schneider and Schweiger, 1991; Shelton et al., 1995). The second IG-like domain is encoded by exon 12 that has been postulated to be the region responsible for binding NTs (Urfer et al., 1995). The transmembrane domain (TMD) of TrkB is encoded by exon 15 and exon 16 encodes the conserved intracellular domain (cICD) shared by TrkB.T1 and TrkB.FL (and includes a stop codon that is part of the TrkB.T1). TrkB.FL has an extended intracellular domain encoded by exons 16–24, containing exon 19 that is an alternative terminating exon which is used in the truncated receptor form (TrkB-T-Shc). Finally, the sixth cluster contain exons 20–24 and encode the tyrosine kinase domain (Middlemas et al., 1991).

The most abundantly expressed TrkB isoforms in the nervous system are TrkB.FL and TrkB.T1 (Klein et al., 1989; Luberg et al., 2010; Stoilov et al., 2002). TrkB.FL is generated from alternative splicing at exon 24, is a 140 kDa (~800 aminoacids) single-pass transmembrane receptor, with an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. TrkB.T1 (95 kDa) is generated from splicing at exon 16 with the same extracellular ligand-binding domain, a transmembrane domain and lacks the intracellular tyrosine kinase domain (Figure 5; Luberg et al., 2010; Stoilov et al., 2002).

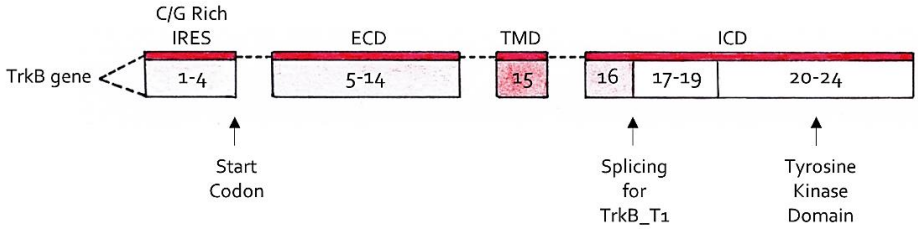

Figure 5. Structure of *TrkB* gene. The *TrkB* gene encodes for at least 36 gene products due to alternative splicing of its 24 exons. Exons 1–4 contain C/G-rich IRES. Exons 5–14 encode the extracellular domain (ECD), exon 15 encodes the transmembrane domain (TMD), and exon 16 encodes the conserved intracellular domain (cICD) shared by TrkB.T1 and TrkB.FL. TrkB.FL has an extended intracellular domain encoded by exons 16–24, where exons 20–24 encode the tyrosine kinase domain. Adapted from Barbara M. Fenner 2012.

The conserved extracellular domains of TrkB.T1 and TrkB.FL contains on the N-terminus one cysteine-rich domain (CC1) separated from the second cysteine-rich domain (CC2) by three consecutive leucine-rich regions (LRR). The LRR domain is essential for high affinity binding and the immunoglobulin-like domain directs ligand-binding specificity (See Figure 3; Haniu et al., 1997; Ninkina et al., 1997). The cytoplasmic domains of TrkB.T1 and TrkB.FL are isotype specific after the first 12 intracellular amino acids. TrkB.FL contains a complex intracellular domain with a tyrosine rich catalytic region (McCarty and Feinstein, 1998) and binding sites for Shc and phospholipase C gamma (PLCγ) among others (Huang and Reichardt, 2001). The short cytoplasmic tail of TrkB.T1 lacks the Shc, tyrosine kinase, and PLCγ regions (Luberg et al., 2010). The truncated cytoplasmic tail prevents TrkB.T1 from having catalytic activity, like TrkB.FL. While it is known that TrkB.T1 can induce intracellular signalling the details of TrkB.T1 signalling are largely unknown (Fenner, 2012).

In the neuromuscular system, both TrkB.FL and TrkB.T1 have been shown to be expressed in neurons, muscle fibre membrane and Schwann cells (Armanini et al., 1995; Funakoshi et al., 1993; Garcia et al., 2010b; Gonzalez et al., 1999). Developing motor neurons express TrkB.FL (Escandón et al., 1994; McKay et al., 1996; Yan et al., 1993), and expression persists into adulthood (Koliatsos et al., 1993; Yan et al.,

1993). The expression of TrkB.T1 in motor neurons increases with age (Armanini et al., 1995; Escandón et al., 1994; Koliatsos et al., 1993), being the predominant isoform expressed in the adult mammalian nervous system (Renn et al., 2009).

TrkB activation by BDNF and downstream signalling

Activation of downstream signalling cascades via TrkB receptor is a multi-phase process. The extracellular domain of TrkB contains 12 consensus N-glycosylation sites (Klein et al., 1989; Watson et al., 1999). N-glycosylation is an important contributor to the structural stability and functionality of TrkB (Haniu et al., 1995) that must be fully glycosylated for insertion into the cell membrane (Watson et al., 1999). The TrkB receptors can be quickly translocated to the plasma membrane from intracellular pools in response to BDNF stimulation or as a result of increased neuronal activity (Du et al., 2000; Meyer-Franke et al., 1998). TrkB can also be inserted to the cell membrane after intracellular transactivation (Puehringer et al., 2013). In this way, BDNF activates TrkB on the cell surface, which leads to the initiation of intracellular signalling cascades and different biological responses.

TrkB.FL

BDNF dimer binding to the TrkB extracellular region promotes receptor dimerization, which activates the intrinsic tyrosine kinase domain, leading to autophosphorylation on specific tyrosine residues in the activation loop (Y701, Y706 and Y707; according to the sequence of TrkB in rat) (Reichardt, 2006). In addition to activating the kinase domain, the phosphorylation of these residues can lead to the transphosphorylation of others tyrosine residues (Cunningham et al., 1997; Friedman and Greene, 1999): Y515 and Y816 an extensively studied phosphorylation sites (Middlemas et al., 1994; Segal et al., 1996). This induce conformational changes to create docking sites for different cytoplasmic molecules and signalling enzymes triggering various parallel signal transduction cascades, with distinct functions. Phosphorylated Y515 and Y816 (pY515 and pY816, respectively) can serve as docking site for Src homology 2 (SH2) adaptor

proteins and phosphotyrosine binding domain containing proteins (PTB) (MacDonald et al., 2000; Qian et al., 1998). pY515 interacts with Shc or Frs2 adaptors and provides a mechanism for the activation of the (i) Ras–mitogen-activated protein kinase (Ras/MAPK) pathway and (ii) phosphatidylinositol-3 kinase –Akt pathway (PI3K/Akt). On the other hand, pY816 links Trk receptors to the (iii) PLCγ pathway (Figure 6; Obermeier et al., 1993). These downstream signalling pathways mediate neuron outgrowth, neuronal differentiation or survival (Pattarawarapan and Burgess, 2003).

The tyrosine phosphorylation that is in close proximity to the C-terminal region of the TrkB receptor, Y816, leads to recruitment and activation of PLCγ (Middlemas et al., 1994; Obermeier et al., 1993) which hydrolyses phosphatidylinositol (4, 5) bisphosphate (PI (4,5) P2) into diacylglycerol (DAG) and inositol tris-phosphate (IP3) (Carpenter and Ji, 1999). IP3 leads to release of calcium from intracellular storages, which in turn activates Ca^{2+}-dependent enzymes such as Ca^{2+}/calmodulin-regulated protein kinases (CaM kinases) and phosphatase calcineurin. In addition, the CaMKII activate the cAMP response element-binding protein (CREB), a transcription factor that recognizes regulatory elements in the bdnf gene, activating its transcription (West et al., 2002). Thus, BDNF binding to TrkB can regulate its own expression. Additionally, the release of Ca^{2+} and the production of DAG also activate different PKC signalling pathways (Canossa et al., 1997; Finkbeiner et al., 1997; West et al., 2001). BDNF acting via TrkB and PLCγ IP3-PKC signalling is important for regulating synaptic transmission, by increasing intracellular calcium (Carmignoto et al., 1997; Levine et al., 1995; Li et al., 1998; Sheng and Kim, 2002).

At the NMJ, TrkB.FL signalling in response to BDNF has been implicated in neurotransmitter release (Garcia et al., 2010a; Kleiman et al., 2000; Knipper et al., 1994; Lohof et al., 1993; Mantilla et al., 2004; Obermeier et al., 1993; Santafé et al., 2001) and in the maintenance of presynaptic and postsynaptic apparatus (Belluardo et al., 2001; Gonzalez et al., 1999; Loeb et al., 2002).

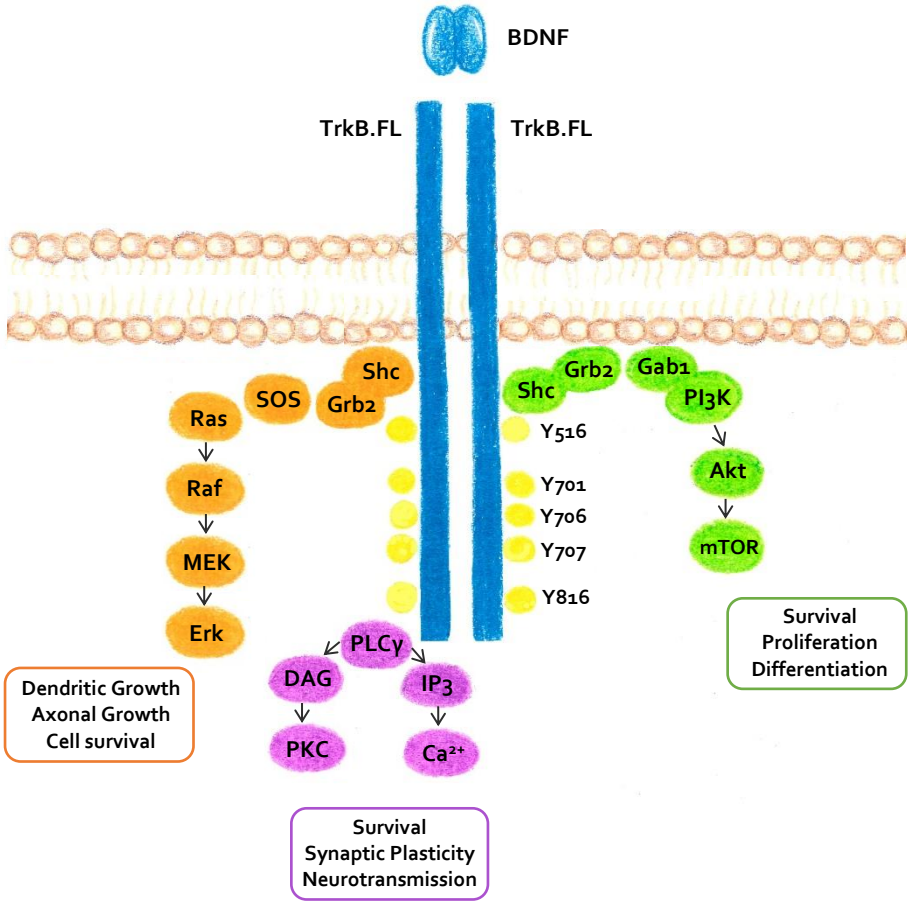

Figure 6. Schematic representation of TrkB.FL signal transduction pathways. The interaction between TrkB.FL and BDNF leads to phosphorylation of a number of tyrosines (Y) in the tyrosine kinase domain. These phosphorylated residues serve as docking sites for cytoplasmic proteins, such as Shc and PLCγ, whose recruitment in turn leads to the activation of the three main intracellular signalling pathways: PLCγ, PI3K/Akt and Ras/MAPK. Through these pathways, BDNF can induce neuronal survival, differentiation, and proliferation. Although not represented in this model, there is extensive cross-talk between these pathways. Adapted from Akil et al., 2016 by P.Cerezuela.

Moreover, contributes to the stabilization of polyinnervated NMJs during the postnatal period of synaptic elimination (Garcia et al., 2010a). In addition, BDNF has been shown to increase motor neuron survival (Henderson et al., 1993; Sendtner et al., 1992; Yan et al., 1992) and promote the axonal growth of motor (Braun et al., 1996) and sensory (Oudega and Hagg, 1999) neurons.

TrkB.T1

The truncated TrkB.T1 receptor can bind and internalize BDNF with the same affinity as TrkB.FL receptors. However, TrkB.T1 lacks the catalytic intracellular tyrosine kinase domain and is therefore incapable of neurotrophic signalling, as TrkB.FL can do it.

TrkB.T1 isoform inhibit the full length TrkB signalling in several ways: (1) function as a dominant negative receptor when form heterodimers with the TrkB.FL (Eide et al., 1996; Haapasalo et al., 2001), (2) sequester BDNF and thus limit and regulate its availability in non-neuronal cells (Biffo et al., 1995; Fryer et al., 1997) and (3) reduce the expression of the TrkB.FL on the membrane (Haapasalo et al., 2002). However, the effects of TrkB.T1 are not just the regulation of TrkB.FL signalling, they can also initiate intracellular signalling through their short intracellular domain in astrocytes (Figure 7; Baxter et al., 1997; Fenner, 2012). TrkB.T1 domain interacts with a Rho GDP dissociation inhibitor (GDI), and binding of BDNF to truncated TrkB.T1 leads to the release of the Rho-GDI to inhibit Rho GTPases. This promotes the rearrangement of the actin cytoskeleton, thus, initiating morphological changes in astrocytic function (Fenner, 2012; Ohira et al., 2005). Moreover, it has been reported an independent role of TrkB.T1 in glia Ca^{2+} signalling. BDNF stimulation causes activation of a G protein that activate PLCγ, IP_3 signalling (independently of TrkB.FL), and promotes Ca^{2+}release from stores and Ca^{2+} entry from the extracellular space (Rose et al., 2003). G protein also induce PKC activation that drives cortical neural stem cells to adopt glial cell phenotypes, regulating its morphology (Cheng et al., 2006).

In the neuromuscular system, the deletion of TrkB.T1 increases NT-dependent activation of downstream signalling targets (e.g., Akt and p70/ sk6) increasing muscle contractility and improving neuromuscular function (Dorsey et al., 2012). Moreover, overexpression of TrkB.T1 induces disassembly postsynaptic receptor clusters at adult NMJ (Gonzalez et al., 1999). Because of their dominant negative function over TrkB.FL, TrkB.T1 can negatively affect the survival role of BDNF (Ninkina et al., 1997).

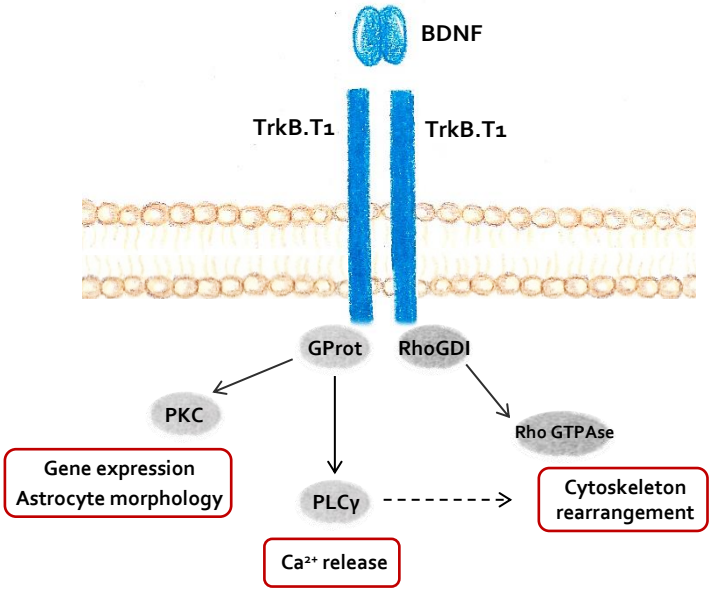

Figure 7. TrkB.T1 intracellular signalling pathway in astrocytes. TrkB.T1 activates G proteins to promote PKC signalling pathways that modulates gene expression and astrocyte morphology (Cheng et al., 2006). G proteins also activates PLCγ that results in Ca^{2+}release from stores and cytoskeletal changes (Rose et al., 2003). Moreover, TrkB.T1 domain interacts with a RhoGDI triggering RhoATPAse activity to induce cytoskeleton rearrangement and filopodia output (Ohira et al., 2005). Adapted from Fenner, 2012 by P. Cerezuela.

Trafficking and intracellular localization of the ligand-receptor complex

After ligation, the NT/receptor complex rapidly activates signalling pathways in the plasma membrane and undergoes internalization. Both processes (insertion into the cell membrane and the internalization of the receptors) are regulated by neuronal activity and increase in intracellular calcium concentration (Du et al., 2003).

The endocytosis of Trk receptors, which occurs by both clathrin dependent and -independent mechanisms, as well as pinocytotic pathways (Shao et al., 2002), is a crucial factor for the cellular localization of activated Trk receptor. NTs-Trk complex is internalized into "signalling endosomes" that are associated with

different signalling adaptors of the Ras-MAP kinase, PLC-γ, and PI3-kinase pathways (Delcroix et al., 2003). After endocytosis, the receptors are thought to converge on peripheral early endosomes also named sorting endosomes (Doherty and McMahon, 2009; Mayor and Pagano, 2007). From there, receptors enter into recycling, retrograde transport, or degradation pathways (Howe and Mobley, 2004) depending on whether the receptors are targeted to recycling endosomes, early endosomes or late endosomes/lysosomes (Di Fiore and De Camilli, 2001; Sorkin and von Zastrow, 2002; Stenmark, 2009; van IJzendoorn, 2006). The dynamics of intracellular trafficking, including through the endo-lysosomal system, are coordinated by Rab GTPases, which are a large family of small GTPases that control membrane identity and vesicle budding, uncoating, motility, and fusion through the recruitment of different and diverse effector proteins (Stenmark, 2009).

TrkB.T1 and TrkB.FL receptors seem to be differentially recycled after BDNF-induced endocytosis with TrkB.FL receptor degraded (targeted to the lysosomes) more quickly than TrkB.T1 (Huang et al., 2009; Stenmark, 2009). In the peripheral nervous system, early endosomes can be transported retrogradely along the axon to the cell body to convey the survival signal. The first observation of retrograde transport of activated Trk receptors was done using sciatic nerve injury, in which the phosphorylated Trk receptors were accumulating in the distal side of the injury indicating that the receptors were transported in clathrin-coated vesicles from the axon terminal towards the soma (Bhattacharyya et al., 1997, 2002). In addition, anterograde transport of TrkB following sciatic nerve injury has been reported (Yano et al., 2001).

The efficiency of endocytosis and the recycling of the receptors back to the cell surface is a mechanism that regulates the availability of the receptors for initiating signalling. Finally, the efficiency of ligand/receptor degradation in late endocytic pathways determine the duration of signalling inside the cell, thus having an

important impact on cellular function (Bronfman et al., 2007, 2014; Miaczynska, 2013; Platta and Stenmark, 2011; Sorkin and von Zastrow, 2002, 2009).

3.3. P75^{NTR} Receptor

In addition to Trk receptors all NTs bind to and activate p75^{NTR} (encoded by *NGFR*) (Rodriguez-Tébar et al., 1990; Rodríguez-Tébar et al., 1992). Most importantly, proneurotrophins bind with much higher affinity than mature NT (Lee et al., 2001), p75^{NTR} is a transmembrane glycoprotein member of the TNF superfamily and regulates a wide range of cellular functions depending with its interaction with co-receptors or NTs. It includes programmed cell death, axonal growth and degeneration, cell proliferation, myelination, and synaptic plasticity (Hempstead, 2002). But also, P75^{NTR} as a monomer can interact with Trk receptors to increase the specificity and the sensitivity to NTs (Bibel et al., 1999; Chao and Hempstead, 1995; Davies et al., 1993). Thus, increasing its signalling leading to neuronal survival, neurite outgrowth, and axonal regeneration.

The receptor is expressed widely in nervous system, with expression in peripheral neurons, in all the cellular components of the neuromuscular system (Cragnolini and Friedman, 2008; Funakoshi et al., 1993; Garcia et al., 2010b).

p75^{NTR} is characterized by four cysteine-rich domains (CRDs) in its extracellular region (See Figure 3; Baldwin and Shooter, 1995; Underwood and Coulson, 2008). In the intracellular portion, in contrast to Trk receptors does not possess kinase activities but comprises death domains that regulate apoptosis and cell death through pro-apoptotic signalling in neurons, oligodendrocytes and Schwann cells, linked to the binding of pro-neurotrophins (pro-NGF, pro-BDNF) (Beattie et al., 2002; Chan et al., 2000; Gentry et al., 2004; Roux and Barker, 2002; Underwood and Coulson, 2008). Indeed, proBDNF binding to Sortilin and to p75^{NTR} can initiate programmed cell death by the association of these two receptors (Nykjaer et al., 2005; Teng et al., 2005). ProBDNF has also been demonstrated to exert rapid morphological effects on NMJ, where proBDNF secreted from myocytes induces

retraction of motor neuron axons, as well as synaptic depression (Je et al., 2012; Yang et al., 2009). *In vivo* experiments using mouse NMJ, showed that proBDNF promotes the synapse elimination through $p75^{NTR}$ and sortilin and mature BDNF supports the survival of the synapses via TrkB (Je et al., 2013). Moreover, proBDNF signalling via the $p75^{NTR}$ mediates the decreased transmission of synapses that are active in an asynchronous manner compared to their neighbouring synapses (Winnubst et al., 2015).

Hence, the cleavage process determines the functional fate of NTs, being able to produce completely opposing functions. Uncleaved forms selectively triggering $p75^{NTR}$ -mediated cell death and mature forms activating either $p75^{NTR}$ or Trk receptors, depending upon the cellular context.

4. Protein Kinase C

PKC family, has emerged as essential for the control of aspects of higher-level signal organization. PKC is a multigene family of serine/threonine kinases that comprises ~2% of the human kinome and are widely distributed in all cells (Lanuza et al., 2014). These proteins can exist both in a soluble state in the cytosol and as a membrane-anchored protein. PKC regulates many cellular processes and are implicated in a wide range of G protein-coupled receptor as well as in other growth factor-dependent cellular responses (Dempsey et al., 2000; Lanuza et al., 2014; Tomàs et al., 2014).

PKC family are more than 14 isoforms and all contain a C-terminal catalytic domain harbouring an ATP binding site (Pearce et al., 2010). The amino-terminal regulatory domain is variable amongst the different isoforms and has a critical role for the specific activity of each isozyme (Figure 8).

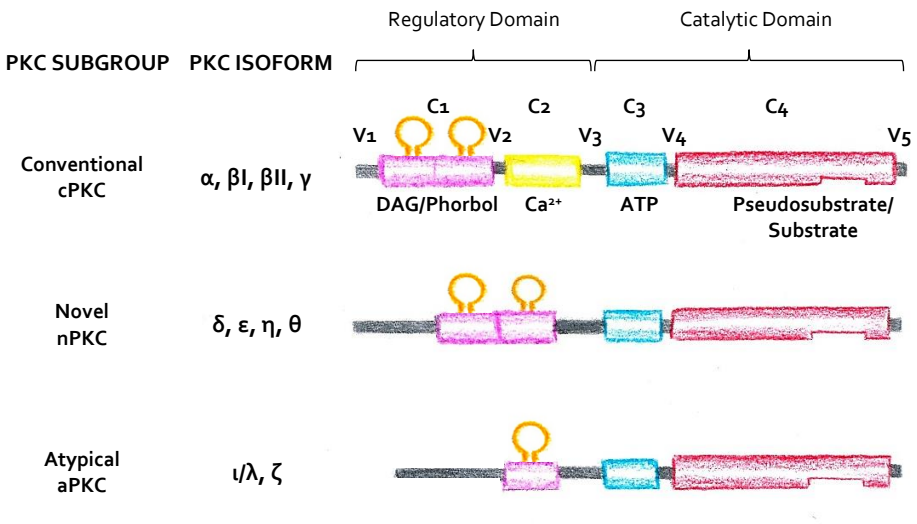

Figure 8. Structure of PKC isoforms. PKC is composed of four conservatives (C1–C4) and five variables (V1–V5) regions. C1 region contains binding sites for DAG, phorbol ester, phosphatidylserine, and the PKC antagonist calphostin C. C2 region contains the binding site for Ca^{2+}. C3 and C4 regions contain binding sites for ATP, some PKC antagonists, and different PKC substrates. Adapted from Khalil, 2010 by P.Cerezuela.

The cloning of the first PKC isoform and its subsequent analysis revealed the existence of four conservative domains (C1-C4, some regulatory and some catalytic) d five variable regions (V1-V5). The regulatory region is like a functional module to bind the membrane and includes the sequence of a pseudosubstrate (in the N-terminal end of C1 domain), and the C1 and C2 domains. The catalytic region contains the kinase domain, the catalytic domain, in which C3 and C4 contain the ATP binding sites and the substrate recognition site to phosphorylate it.

PKC isoforms are classified into three structurally and functionally groups specified by their divergent regulatory domains (Figure 8).

The conventional or classical PKCs (cPKCs). α, βI, βII, and γ have the four conserved regions (C1–C4) and the five variable regions (V1–V5). This family is activated by a combination of DAG and phospholipid (as Phosphatidylserine; PS) binding to their conserved region 1 (C1) which is presented duplicated as a tandem

(C1A and C1B) in cPKC and nPKC isozymes. And moreover, by Ca^{2+}, a soluble ligand that binds to the C2 domain. Both classic and novel PKC families are targets of the tumour promoter PMA (phorbol-12-myristate-13-acetate) binding to the C1 domain, which has been used extensively in the study of PKC function.

The novel PKCs (nPKCs), which include PKCδ, PKCε, PKCθ and PKCη, are activated by DAG and phospholipids (as PS) so they have the C1 domain. But they do not respond directly to Ca^{2+} (Mellor and Parker, 1998) thus, lacking the C2 region.

The atypical PKCs (aPKCs) ζ and λ/ι have only one cysteine-rich zinc finger-like motif and are dependent on PS, but not affected by DAG, phorbol esters, or Ca^{2+}.

When inactive, PKC is auto-inhibited by a pseudosubstrate sequence (in the N-terminal end of C1 domain), which occupies the substrate binding pocket (Pears et al., 1990). PKC activation occurs when second messengers and/or allosteric effectors bind to its regulatory domain, resulting in the release of the pseudosubstrate sequence and the substrate binding and phosphorylation (Figure 9; Dutil et al., 2000).

Models of PKC activation have generally focused on the intramolecular interaction between the pseudosubstrate domain and the catalytic pocket. However, there is a second intramolecular interaction that is based upon PKC interactions with receptors for activated C kinase (RACKs), a family of membrane-associated PKC anchoring proteins. They act as molecular scaffolds to localize individual PKCs to distinct membrane microdomains in close proximity with their allosteric activators and unique intracellular substrates. It has been proposed that cells express a unique RACK (with a distinct subcellular localization) for each PKC isoform and that PKC-RACK interactions are essential for isoform-specific cellular responses (Daria Mochly-Rosen et al. 1991; Mochly-Rosen & Gordon 1998).

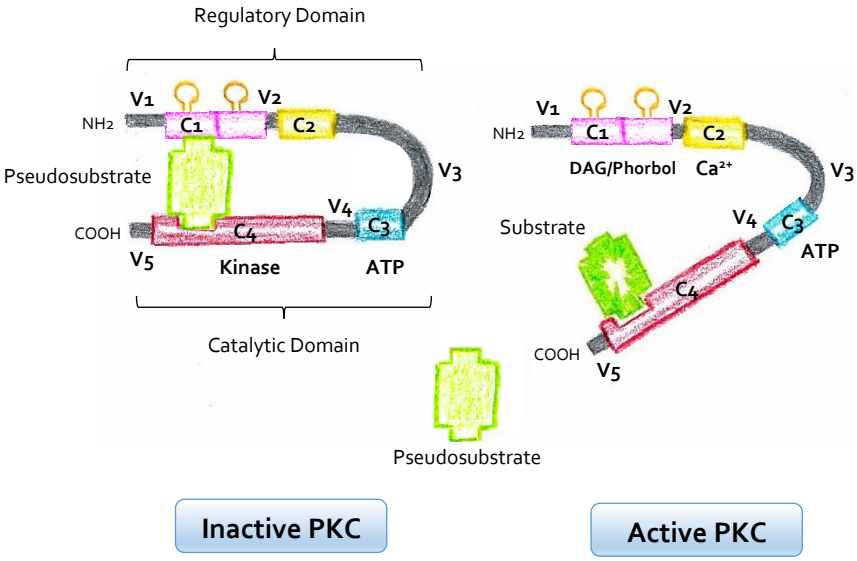

Figure 9. Mechanisms of PKC activation. In the inactive conformation, PKC is folded in such a way to have an endogenous pseudosubstrate bind to the kinase region. PKC activation takes place when PS, DAG or phorbol ester, and Ca^{2+} allows PKC to be unfolded. In this active conformation, the true substrate can bind to the kinase region. In the presence of ATP, PKC causes phosphorylation of its substrate. Adapted from Khalil, 2010 by P.Cerezuela.

4.1. Mechanisms of activation and inactivation of cPKC

The mechanisms involved in PKC activation have been extensively studied (Newton, 2003, 2010; Parekh et al., 2000) and it is required different steps before is able to phosphorylate its substrates. After activation, PKC is inactivated by degradation.

Maturation process: transphosphorylation and autophosphorylation

PKCs undergo a process of maturation before the enzyme is able to become activated (Newton, 2003; Parekh et al., 2000). PKC maturation includes a series of phosphorylation steps that involves the three key phosphorylation sites on the C-terminus known as the activation-loop (Aloop), the turn-motif (TM) and the hydrophobic-motif (HM). All three sites are conserved in cPKCs and nPKCs, but the

aPKCs do not contain an HM residue but instead possess a negatively charged glutamic acid, perhaps mimicking a constitutively phosphorylated state (Keranen et al., 1995). The first step is mediated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and occurs at the Aloop site. After PDK1 phosphorylation, the TM and the HM are exposed in the C-terminal domain and autophosphorylation takes place leading to stabilization of the enzyme. However, other studies suggest that after the phosphorylation of the Aloop by PDK1, then TM and HM are phosphorylated by mTORC2 (Facchinetti et al., 2008; Ikenoue et al., 2008). The mature PKC, now 'primed' for activation by DAG and Ca^{2+}, is released into the cytosol and kept in an inactive conformation by intramolecular interactions between the N-terminal pseudosubstrate region and the kinase domain (Griner and Kazanietz, 2007; Oancea and Meyer, 1998; Violin et al., 2003).

Recent finding shows that PKC phosphorylation on sites such as the Aloop may occur much later during their life cycle and predominantly in response to cellular stimulation. Therefore, PKCs can also exist in non/hypophosphorylated forms, with cellular stimulation resulting in inducible phosphorylation at some or all of these sites. For example, inducible phosphorylation has been reported for cPKCs following stimulation (Freeley et al., 2011; Osto et al., 2008; Wang et al., 2007; Zhou et al., 2003).

Activation process: binding of Ca^{2+} and DAG

Once that intracellular Ca^{2+} and DAG increase, classical PKCs are tethered to the membrane through calcium binding to the C2 domain, where it binds anionic phospholipids. Once engaged on the membrane, DAG binding to the C1 domain confers a high-affinity interaction between PKC and the membrane, an interaction that is enhanced by stereospecific binding to PS. The coordinated engagement of both the C1 and C2 domains on the membrane provides the energy to perform a massive conformational change that releases the pseudosubstrate domain from the substrate-binding site (Figure 9). This allows for substrate binding, phosphorylation and the activation of downstream signalling effectors (Colón-

González and Kazanietz, 2006; Griner and Kazanietz, 2007). Novel PKCs might translocate to membranes slower than classical PKCs because they are not pre-targeted by calcium, but this is compensated for by an increased affinity for DAG (Giorgione et al., 2006).

Inactivation: proteolysis and degradation

After their activation, PKC is downregulated through a poorly understood mechanism. The short half-life of DAG is probably key for reversing the activation of PKC which results in the translocation of cPKCs-nPKCs back to the cytoplasm. Prolonged PKC signalling that is promoted by phorbol esters or sustained DAG signalling results in the downregulation of PKCs (Freeley et al., 2011; Lee et al., 1996; Leontieva and Black, 2004; Lu et al., 1998; Newton, 2010). At least four potential mechanisms that are not mutually exclusive have been proposed for the down-regulation of PKC.

The first is a conformation-dependent protease sensitivity of PKC. Based on the in vitro sensitivity of PKC to various proteases, Ca^{2+}-activated neutral proteases (such as calpains) were hypothesized to degrade membrane-bound PKC (Pontremoli et al., 1988). Down-regulation of PKCα and δ in membrane fractions was partially blocked by calpain inhibitor II in muscle cells (Hong et al., 1995). The second possibility is a vesicle-dependent, PKC activity-dependent PKC degradation. When mammalian PKCs were expressed in fission yeast and activated with phorbol ester treatment, increased vesicle traffic was noted (Goode et al., 1994). It has been hypothesized that association of PKCs with the vesicles may lead to their targeting to lysosomes or some other subcellular compartment where they are degraded (Parker et al., 1995). The third potential mechanism is a proteasome-dependent degradation involving ubiquitination. In human fibroblasts, PKCα and ε were ubiquitinated following activation with phorbol ester or with ligands for certain cell surface receptors and then degraded by proteasomes (Lee et al., 1997). Finally, proteolysis of PKC during apoptosis has suggested that PKC may be cleaved by a caspase as part of an apoptosis pathway (Datta et al., 1997).

4.2. PKC function at the NMJ

The specific cofactor requirements, tissue distribution and cellular compartmentalization suggest differential functions and fine tuning of specific signalling cascades for each isoform (Mochly-Rosen et al., 2012). Thus, specific stimuli can lead to differential responses via isoform specific PKC signalling regulated by their expression, localization and phosphorylation status in particular biological settings.

Several PKC isoforms, differently regulated, have been localized at the NMJ (Besalduch et al., 2010; Obis et al., 2015a). Specifically, PKCα, βI, ε, θ, λ, ζ are localized in the motor nerve terminals; PKCα, βII, θ, λ, ζ are localized in the postsynaptic component of the NMJ and PKCα, βII, λ, ζ are also localized in the perisynaptic Schwann cells. This wide distribution of PKC isoforms at the NMJ, with several isoforms present within a single cell, suggests that each mediates specific intracellular functions.

Members of each group of PKC isoforms have been implicated in various cellular phenomena (Besalduch et al., 2013; Hilgenberg and Miles, 1995; Lanuza et al., 2000; Li et al., 2004; Perkins et al., 2001). During development, PKC activity is also involved in synaptic elimination process due to the blockade of PKC by calphostin C (CaC) that increases the number of polyinnervated axons, allowing a delay in synaptic elimination process. In the paradigmatic adult NMJ, whereas protein kinase A (PKA) is tonically coupled to potentiate ACh release, PKC couples in a regulated manner when several activity demands are imposed (Besalduch et al., 2010; Santafé et al., 2005, 2006, 2009). In particular, it is known that nPKCε coupling is clearly involved to maintain or potentiate ACh release in the NMJ in several conditions (i.e. low frequency electrical stimulation-induced, high external Ca^{2+} and inflow and PKA stimulation, among other; Obis et al., 2015). This isoform, then, may be involved in the presynaptic function of maintaining and potentiating transmitter release, probably by controlling the coupling of other PKC isoforms to the ACh release.

In the presynaptic membrane, mAChRs are an important self-control mechanism of ACh release and PKC is also related with it. Specifically, blocking the muscarinic mechanism results in a CaC-inhibitable PKC coupling and ACh release potentiation. Thus, PKC become coupled on mAChR signalling inhibition (Santafé et al., 2006). Moreover, there is also functional evidence indicating that there is an interaction between TrkB receptors and PKC to modulate neurotransmission in NMJ (Santafé et al., 2014).

Recently, in our laboratory it has been demonstrated that two exclusively PKC isoforms located at the nerve terminal of the NMJ (cPKCβI and nPKCε) are modulated by pre-synaptic and nerve-induced muscle contraction (Besalduch et al., 2010; Obis et al., 2015a). The results suggest that the muscle cell contraction-dependent increase in the presynaptic isoforms (βI, α and ε) in the synaptic zone may require neurotrophic positive feedback from the postsynaptic component as a result of the postsynaptic contractile activity.

5. Phosphoinositide-dependent protein kinase 1 (PDK1)

The discovery of the PDK1 as the upstream kinase for PKC represented an important step to understand PKC regulation (Calleja et al., 2014; Cenni et al., 2002; Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998). It was first purified from tissue extracts as an enzyme that could phosphorylate the T-loop of PKB (Thr308) in the presence of PI(3,4,5)P_3 (Alessi et al., 1997a; Stokoe et al., 1997).

PDK1 is a Ser/Thr kinase with a catalytic domain near its N-terminal and a pleckstrin homology (PH) domain at its C-terminal (Figure 10). PDK1 with its PH domain binds to either PI(3,4,5)P_3 and PI(3,4)P_2 (produced by phosphatidylinositide 3-kinase; PI3K) and to other phosphoinositides such us PI(4,5)P_2 (Alessi et al., 1997a; Currie et al., 1999). This binding is necessary for targeting PDK1 to the plasma membrane. Here, is optimal for activation (Yang et al., 2002a, 2002b) to interact with and

phosphorylate its substrates such as PKC (Balendran et al., 2000; Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998).

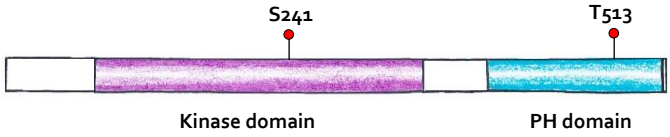

Figure 10. Schematic representation of PDK1 structure. PDK1 protein containing a PH domain C-terminal and its kinase domain. The major autophosphorylation sites critical for PDK1 activation are in red circles. Adapted from Calleja et al, 2014 by P. Cerezuela.

PDK1, like all other AGC kinases, requires phosphorylation at its Aloop residue Ser241 in order to be activated (Casamayor et al., 1999; Wick et al., 2002). It is ubiquitously expressed in cells and, surprisingly for an enzyme that regulates as much as 23 agonist-stimulated AGC kinases, its own catalytic activity is not stimulated by these agonists (Alessi et al., 1997b). Several regulatory mechanisms and phosphorylation sites have been proposed to contribute to the regulation of PDK1 activity (Casamayor et al., 1999; Riojas et al., 2006; Wick et al., 2003; Yang et al., 2008). The finding that bacterially expressed PDK1 is fully active, indicated that PDK1 is autophosphorylated at its own Ser241 Aloop residue, perhaps explaining why it is constitutively active in mammalian cells. Evidences indicated that PDK1 autophosphorylation at Ser241, is mediated by an intermolecular *trans* reaction, rather than intramolecular *cis* reaction (Wick et al., 2003). Much research has focussed on understanding how an enzyme that is always active is then capable of phosphorylating in an inducible manner its myriad of substrates in response to specific stimuli.

Different substrates of PDK1 are phosphorylated and activated by distinct regulatory mechanisms. The role of phosphorylation on the activity and stability of cPKCs has been studied extensively and previously reviewed (Newton, 2003; Parekh et al., 2000). The studies suggest that PKC Aloop phosphorylation is necessary for activity and required for further phosphorylation of the TM and HM

site, which are required for stability. A-loop phosphorylation by PDK1 is, therefore, indirectly required for the stability of conventional PKC isoforms. The activity of PDK1 toward conventional PKCs has been shown to be independent of phosphoinositides (Sonnenburg et al., 2001). Rather, the conformation of PKCs controls its phosphorylation by PDK1 (Newton, 2010): when PKC is in an open conformation, the pseudosubstrate is removed from the substrate-binding cavity, thus unmasking the Aloop site to allow phosphorylation by PDK1 (Dutil and Newton, 2000). As described above, maturation of PKC requires priming phosphorylation by PDK1 at Aloop, but once PKC is further phosphorylated at the TM and HM site, phosphorylation at Aloop becomes indispensable, at least for cPKCβII (Dutil and Newton, 2000). Indeed, this site is dephosphorylated in a serum-sensitive manner such that only about one-half of the pool of cPKC in cells cultured in serum is phosphorylated on the Aloop, yet is quantitatively phosphorylated at the TM and HM sites (Keranen et al., 1995; Newton, 2010; Sonnenburg et al., 2001).

A particular feature of cPKCs is that dephosphorylation causes aggregation, which, in PKCα, parallels the neutral detergent insolubility of the dephosphorylated forms that can accumulate in vivo (Bornancin and Parker, 1996; Parekh et al., 2000). The instability of dephosphorylated PKC has also been verified in PDK1 knockout embryonic cells, in which PKCs are not phosphorylated by PDK1 and, as a result, their levels are vastly reduced (Balendran et al., 2000; Williams et al., 2000). Taken together, the results indicate that phosphorylation by PDK1 is required for the correct folding of PKCs in vitro and in vivo (Biondi, 2004).

Although the importance of PDK1 in PKC signalling has been well characterized, the regulatory mechanism of PDK1 activity is controversial and whether PDK1 is modulated by synaptic activity in the NMJ remains unknown.

6. Neuromuscular disorders: Amyotrophic lateral sclerosis

Neuromuscular disorder is a broad term that encompasses many different syndromes and diseases that either directly or indirectly impair the function of the skeletal muscles.

One of the most common neuromuscular disorders is amyotrophic lateral sclerosis (ALS) whose onset is in the adulthood, usually after the age of 50. It has a prevalence of about 2 people per 100.000 worldwide and is slightly more frequent in men. ALS is a progressive neurodegenerative disorder characterized by the selective degeneration of the upper motoneurons in the cerebral cortex and/or lower motoneurons in the brainstem and spinal cord which causes muscular weakness, atrophy and spasticity among other symptoms (Kiernan et al., 2011; National Institute of Neurological Disorders and Stroke, 2017). It is a good example in which the progressive interruption, at the neuromuscular junction level, of the connection between nerve and muscle leads to the pathological non-communication of the two tissues.

By the moment, despite of different new therapies based on stem cells, different physical, nutritional and speaking therapies are the most useful techniques to ameliorate life quality of ALS patients and further extensive research should be done (National Institute of Neurological Disorders and Stroke, 2017).

About 90% of ALS cases are sporadic and the remaining 10% of ALS cases are familial (FALS). At least 10 different loci (ALS1-10) have been suggested to cause a "pure" ALS phenotype by genetic linkage, and disease-causing mutations have been described for seven of these (*SOD1* [ALS1], *Alsin* [ALS 2], *SETX* [ALS 4], FUS [ALS 6], VAPB [ALS 8], *ANG* [ALS 9] and TARDBP [ALS 10]; Valdmanis et al., 2009). In about 20% of FALS cases, the cause can be attributed to a mutation in the Cu^{2+}/Zn^{2+} superoxide dismutase 1 (SOD1), a ubiquitously-expressed free-radical defence enzyme (Rosen et al., 1993). The mutations cause misfolding of this normally stable homodimeric protein (Zheng et al., 2004) but the exact mode of action of mutant SOD1 remains unclear. Multiple possibly interrelated mechanisms have been postulated that cause MNs death: toxic intracellular aggregation of

mutant SOD1, oxidative damage, mitochondrial dysfunction, RNA binding and destabilization, alterations in axonal transport, growth factor deficiency, and glutamate excitotoxicity (Rothstein, 2009). Overexpression of mutant forms of human SOD1 such as SOD1 (G93A) in transgenic mice mimics human ALS disease symptoms and progression (Gurney et al., 1994). To date, SOD1 (G93A) is one of the best characterized mouse models to study ALS.

It is widely accepted that ALS is caused by MN degeneration. However, NMJ degeneration precedes and may even directly cause MN loss. Many mechanisms explaining NMJ degeneration have been proposed such as the disruption of anterograde/retrograde axonal transport, irregular cellular metabolism, and changes in muscle gene and protein expression (Cleveland and Williamson, 1999; Zhang et al., 1997). A major hypothesis in the latest ALS research is the "distal axonopathy" with pathological changes occurring at the NMJ, at very early stages of the disease, prior to motoneurons degeneration and onset of clinical symptoms (Moloney et al., 2014). Morphological alterations of NMJs are present since an early-stage of the disease and may significantly contribute to functional motor impairment in ALS patients that result in muscle atrophy and paralysis. Therefore, the loss of normal NMJ may be the cause of motoneuron death due to the loss of nerve-muscle contact and the resulting disruption of the neurotrophic and neuromotor control. It has been demonstrated that neuromuscular transmission is impaired in presymptomatic (4–6 weeks old) ALS mice compared to age-matched controls (Rocha et al., 2013), which is a sign of NMJ destruction. Moreover, other electrophysiological changes in electrical excitability or conduction velocity disrupt the necessary interactions between the nerve terminals, muscle fibres and perisynaptic Schwann cells required for appropriate NMJ formation and this leads to a delay in maturation of the NMJ in presymptomatic animals (Blijham et al., 2007; Caillol et al., 2012; van Zundert et al., 2008). Therefore, the abnormal NMJ function of presymptomatic ALS mice could lead to a destabilization of it and finally of MN. Because of that, maintaining neuromuscular activity in partially

denervated muscles extends motor units survival time over the course of the disease (Gordon et al., 2010).

It is important to note that studies in the mouse model SOD1(G93A) has shown that ALS disease preferentially involves specific MN and muscle fibre types. There is a pattern of preferential loss of larger MNs innervating faster muscle fibres. Hegedus and colleagues suggested that MNs innervating the slower muscle fibres are more resistant than those innervating the faster ones (Hegedus et al., 2007, 2008). Moreover, it is described that in the fast-twich muscles plantaris and tibialis, only the fast IIB fibres were atrophied in ALS mice. In addition, these muscles suffered a significant fast-to slow transition from fast-twitch type II fibres to slow-twitch type I fibres and, within the type II fibre population, from type IIB/IIX to IIA fibres (Deforges et al., 2009).

Training exercise has been proposed to provide a beneficial therapy during the early or late stages of ALS (Drory et al., 2001; Pinto et al., 1999). The beneficial effects are based on the cellular adaptations induced by training exercise in the brain, spinal cord, and skeletal muscles that could counteract the oxidative stress complication in ALS. For instance, training exercise increases the capacity of antioxidant enzymes and reduces lipid peroxides in brain regions of rats (Husain and Somani, 1997). In skeletal muscle, training reduces oxidative stress following exercise (Miyazaki et al., 2001), increases the mitochondrial capacity (Holloszy et al., 1970), and increases the expression of neurotrophic factors, as BDNF (Gómez-Pinilla et al., 2001). The latter could be particularly beneficial in ALS because neurotrophic factors could prevent MN degeneration, preserve muscle innervation, and inhibit muscle atrophy (Acsadi et al., 2002; Elbasiouny and Schuster, 2011; Manabe et al., 2002; Sun et al., 2002). However, over the last decades, several evidences have supported the administration of neurotrophic factors to ameliorate the ALS disease process, but with limited success, possible due to the consequent activation of $p75^{NTR}$, implicated in ALS (Henriques et al., 2010; Krakora et al., 2012). There are also studies that refer that the practice of physical exercise has barely effective (Drory et al., 2001) or even negative effects (Harwood et al., 2016) for ALS

patients arguing that muscles affected by ALS do not have enough resistance to oxidative stress and practicing physical exercise would even aggravate the situation. Thus, it is important take into account the type of exercise and how it impacts in ALS disease. Deforges et al. (2009) reported for the first time, that exercise impacts on all neural cell distribution in ALS spinal cord with an efficiency dependent on the nature of the exercise.

In summary, all the molecular changes occurring at the NMJ, independently of its origin, influence the stability of the synapse during the course of ALS. For that reason, it is essential to know how inducers of instability and protectors of NMJ integrity are balanced to maintain the appropriate structure and function of the NMJ.

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B. HYPOTHESIS AND OBJECTIVES

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HYPOTHESIS AND OBJECTIVES

■ HYPOTHESIS

Previous studies suggested the existence of a retrograde factor influenced by synaptic activity-induced muscle contraction that could modulate presynaptic PKC at the NMJ (Besalduch et al., 2010; Obis et al., 2015a).

We hypothesize that synaptic activity and muscle contraction are closely coordinated to regulate: (1) BDNF/TrkB signalling pathway modulating presynaptic cPKC isoforms (α, βI) to balance synaptic function and (2) PDK1 activation to maturate cPKC in skeletal muscle. In concordance with that, a decreased neuromuscular activity in ALS model mice, changes BDNF levels and induces modifications in its receptors. Moreover, an increased neuromuscular activity induced by exercise prevents the ALS-induced modifications in BDNF signalling.

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OBJECTIVES CHAPTER I

■ GENERAL OBJECTIVE

To investigate the involvement of synaptic activity and muscle contraction in (1) BDNF/TrkB signalling pathway modulating presynaptic cPKC isoforms (α, βI) to control neurotransmission and (2) PDK1 activation to maturate cPKC in skeletal muscle.

■ SPECIFIC OBJECTIVES

1. To determine whether synaptic activity and muscle contraction affect proBDNF and mBDNF protein levels in the skeletal muscle.
2. To determine whether BDNF receptors, TrkB.FL (and its phosphorylation), TrkB.T1 and $p75^{NTR}$ are modulated by synaptic activity and muscle contraction in the skeletal muscle.
3. To determine whether cPKCα and cPKCβI (and their phosphorylation), are modulated by synaptic activity and muscle contraction in skeletal muscle. Moreover, to determine whether they are regulated by the activity-induced action of BDNF/TrkB signalling.
4. To localize PDK1 in the NMJ.
5. To determine whether PDK1 (and its phosphorylation) is modulated by synaptic activity and muscle contraction in skeletal muscle.
6. To specify whether PDK1 and cPKCβ (and their phosphorylation) are modulated by synaptic activity and muscle contraction in the cytosol and membrane fraction of skeletal muscle.
7. To determine whether cPKCβI is involved in neurotransmission.

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OBJECTIVES CHAPTER II

This Chapter has been done in collaboration with the Neuromuscular degeneration and plasticity team (UMR-S 1124) in Paris Descartes University (France) and it was possible thanks to Prof. Frédéric Charbonnier and Dr. Olivier Biondi. All training protocols were carry out in their laboratory and then, plantaris muscles were sent to Rovira i Virgili University where muscles were analysed by Western Blot.

■ GENERAL OBJECTIVE

To characterize whether BDNF and its receptors (TrkB and $p75^{NTR}$) are affected in ALS disease and whether the modulation of neuromuscular activity by exercise in a pathological context like ALS, could affect the levels of BDNF, TrkB and $p75^{NTR}$.

■ SPECIFIC OBJECTIVES

1. To determine whether BDNF protein levels are affected in skeletal muscle of ALS mice (SOD1G93A) and whether they could be modulated by two different types of physical exercise (running and swimming-based training).
2. To determine whether TrkB.FL (and its phosphorylation), TrkB.T1 and $p75^{NTR}$ are affected in skeletal muscle of ALS mice (SOD1G93A) and whether they could be modulated by two different types of physical exercise (running and swimming-based training).
3. To determine whether running and swimming-based training promotes different effects in ALS mice.

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C. METHODOLOGY

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METHODOLOGY

Animals

Sprague-Dawley rats

To perform the first Chapter of this thesis, we used Sprague-Dawley rats of 40–50 days (Criffa, Barcelona, Spain). The animals were cared for in accordance with the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All the procedures realized were reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili (URV; Reference number: 0289) in accordance with *Llei 5/1995 and Decret 214/1997* of *Generalitat de Catalunya*. All rats were maintained on the animal facility in standard cages under standards conditions: constant temperature (22º±2ºC), relative humidity (50±10%) and a 12-hour light/dark schedule. Standard rodent chow and clean water were available *ad libitum*.

To carry out the experimental procedures, animals were anesthetized with 2% tribromoethanol (TBE). Once the animal was deeply anesthetized (lacking the reflexes), we proceeded to the handling and their sacrifice by making a cut in the jugular vein. As fast as possible, started the dissection procedure avoiding the degradation processes. At least five independent animals (n > 5) were used to evaluate the following techniques.

SOD1 G93A mice model

The mouse line employed in the Chapter II of the present work is the SOD1-G93A transgenic mice (also called B6SJL.SOD1-G93A). It has been extensively characterized to study ALS. These transgenic mice express high levels of the transgene (up to 25 copies) composed of the human SOD1 gene carrying a glycine to alanine transition in the position 93 (G93A mutation) (Chiu et al., 1995). The mutation induces misfolded proteins that form aggregates with toxic proprieties and cause the loss of function of other proteins. Moreover, those aggregates induce

the collapse of the proteasome, the depletion of chaperones and the dysfunction of organelles such as mitochondria which increases oxidative stress (Boillée et al., 2006). Similar to ALS patients, SOD1-G93A mice, develop muscle weakness and atrophy along with symptoms such as spasticity, limb-grasping and clonus. According to Chiu and colleagues (1995) mice reached the humane end point at 136 days of age, which was defined as 10 % loss of body weight or inability to right themselves within 30 seconds when placed on their side. Further pathological studies revealed that, as occurs in ALS patients, these mice have a progressive loss of MNs starting at 100 days of age. By end stage, a 50 % loss of MNs is detected in the spinal cord and concretely, there is a pattern of preferential loss of larger MNs innervating faster muscle fibres (Chiu et al., 1995; Fischer et al., 2004; Gould et al., 2006; Hegedus et al., 2007, 2008). Electrophysiological studies confirmed the behavioural data showing a loss of muscle strength from 40 days of age. Events of denervation and reinnervation of skeletal muscles are detected from 47 days of age, moreover evidence of axonal degeneration is present in ventral roots from 80 days of age (Chiu et al., 1995; Fischer et al., 2004).

The SOD1 mouse model remained for ~15 years the primary rodent model used for studying ALS, as SOD1 mutations represented until then the most frequent known genetic cause of familial ALS (~20% of familial cases).

Transgenic male mice with the G93A human SOD1 mutation B6SJL-Tg (SOD1-G93A) 1Gur/J (ALS mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). B6SJL male mice (Janvier, le Genest, France) served as a control for this mutant strain. Animals were cared in accordance with the *Ministere de la Recherche et de la Technologie* (France) guidelines for the detention, use and ethical treatment of laboratory animals. All mice were kept on the animal facility under standards conditions: constant temperature (22º±2ºC), relative humidity (50±10%) and a 12-hour light/dark schedule. All experimental procedures which included minimizing the number of animals used and their suffering were approved by the policies of the French Agriculture and Forestry Ministry. Disease onset was defined as the time corresponding to the first observation of myotonia symptoms in the

mice hind limb. Death was scored when mice were unable to stand on their feet 30 seconds after having been placed on their side. It is referred from now as ALS mice.

The study of NMJ: skeletal muscles

Skeletal NMJ have been used for many years for the study of neurotransmission, synapsis structure and plasticity. It is an ideal model because they are highly accessible, relatively simple, functionally uniform, and so much larger than central synapses that their size and shape can be assessed light microscopically (Sanes and Lichtman, 1999).

Levator auris longus

Levator auris longus (LAL) muscle was described by Denise Angaut-Petit et al. (1987) and it is located under the dorsal skin in the area of the head and neck allowing the movement of the ears (Figure 11). It contains cranial and caudal portions that originate from the midline of the cranium and extend laterally to the cartilaginous portion of each pinna. It is innervated by facial nerve and consists of fast-twitch muscle fibres (Erzen et al., 2000) which are arranged in five or six layers of cells in the cranial portion (5.25 ± 0.78, Lanuza et al., 2001).

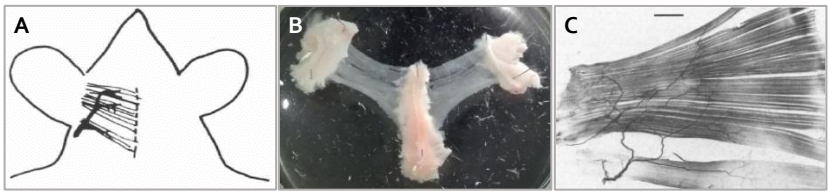


Figure 11. Schematic representation of levator auris longus muscle. (A) LAL muscle as it appears when the skin is removed on the dorsal aspect of the head and neck. **(B)** Dissected LAL muscle of adult rat placed in a Petri dish with Sylgard. **(C)** Whole mount preparation showing the distribution of the nerve (silver impregnation). Bar represents 1.2 mm in (C). (A) and (C), adapted from Angaut-Petit et al., 1987.

We used LAL muscle to perform immunohistochemistry (IHC) technique offering advantages to study the NMJ model because of its thinness and its superficial location. First, offers a good visibility of the global facial nerve endings without having to make sections (Lanuza et al., 2001, 2014). Second, antibodies can easily penetrate the muscle allowing to perform high quality imaging in IHC technique. Third, a small applied volume (˜50µl) easily covers the entire muscle surface and permits visualization and analysis of almost all the NMJs within the muscle. Last, its easy removal and handling permits the maintenance of the physiological conditions as long as possible.

Diaphragm muscle

The diaphragm is a thin, elliptically shaped muscular structure separating the thoracic and abdominal cavities (Figure 12). It is the primary muscle of the inspiratory pump and it can be anatomically divided into 2 hemidiaphragms (right and left). Together with the rib cage and abdomen it gives rise to the chest wall. It receives its entire innervation from the phrenic nerve and it is a slow muscle in large mammals and a fast muscle in rat and mouse (predominantly type IIX fibres; Schiaffino et al., 1989). Nerve stimulation simultaneously recruits all motor units and uniforms the heterogeneous level of activity of the fibres. In this thesis, diaphragm muscle was used to perform mRNA and protein analysis (qRT-PCR, ELISA and Western Blot after stimulation protocols), immunohistochemical (IHC) and functional analysis (electrophysiology). It has been described as a useful model of flat, thin muscle (typically 10-15 fibres thick) to study synaptic function (Besalduch et al., 2010; Chand et al., 2015; Obis et al., 2015a; Rosato Siri and Uchitel, 1999; Urbano et al., 2003). The good visibility of the individual muscle fibres, motor axons and terminals allows accurate electrophysiological studies of presynaptic signals. The size of the muscle allows to collect a fair amount of sample for WB procedures and its anatomic division in two hemidiaphragms allows to perform the experimental condition in one hemidiaphragm and use the other as its control. Moreover, the length of the phrenic nerve and its accessibility help to

dissect and stimulate it and thus study independently synaptic activity and muscle contraction.

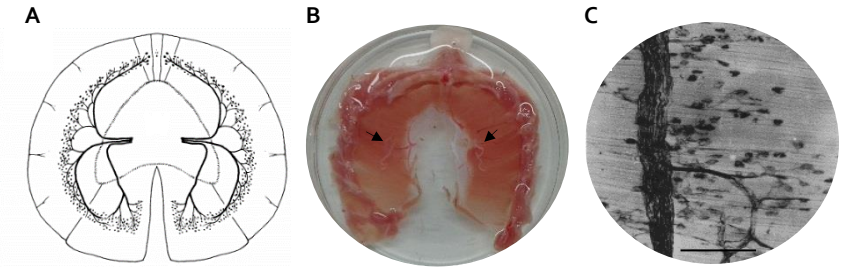


Figure 12. Schematic representation of diaphragm muscle. (A) Rodent diaphragm structure with the phrenic nerve distribution. **(B)** Two hemidiaphragms of adult rat placed in a Petri dish with Sylgard with the phrenic nerve (arrows). **(C)** Whole mount preparation showing the nerve branch supplying a band of motor end plates (cholinesterase-silver). Bar represents 200 μm in (C). (A) and (C), adapted from Comerford and Fitzgerald, 1986.

Plantaris muscle

The plantaris muscle consists of a small, thin muscle belly, that courses along the posterior aspect of the leg as part of the posterosuperficial compartment of the calf (Figure 13). It is a two-joint muscle, meaning its action can influence both joints involved. It contributes to ankle flexion if the foot is free, or to bending the knee if the foot is fixed. Due to the high density of muscle spindles within the muscle, the plantaris is thought to be an organ of proprioceptive function for the larger, more powerful plantarflexors such as the triceps surae muscle (Spina, 2007).

We used plantaris muscle to perform Western Blot analysis after training protocols in ALS mice. It is a fast-twich muscle that is comprised mostly of faster fibre types (IIX ≈ 41%, IIX/IIB ≈ 28%, and IIB ≈ 20% in mice; Denies et al., 2014). As ALS preferentially involves loss of larger MNs innervating faster muscle fibres, plantaris muscle is a good model to attempt a first approach at studying skeletal muscle degeneration in ALS (Chapter II).

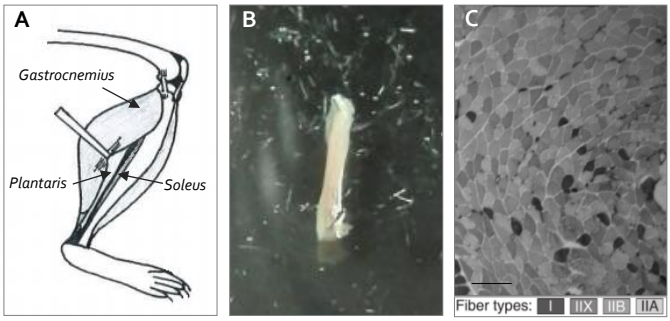


Figure 13. Schematic representation of plantaris muscle. (A) Anatomical location of plantaris muscle in mouse hindlimbs**. (B)** Dissected plantaris muscle of mouse placed in a Petri dish with Sylgard. **(C)** Myofibrillar ATPase staining showing the different fibre types in the plantaris muscle (containing a mixture of fast fibre types) (Denies et al., 2014). Bar represents 100 μm in (C).

Antibodies

Table 1 provides an overview of the antibodies and neurotoxins used in this thesis and the corresponding method.

Antigen	Conjugate	Host	Dilution	Source	Method
AChR (α-BTX)	TRITC		1/800	Molecular probes	IHC
BDNF		Rabbit	1/100	Santa Cruz	WB
GAPDH		Goat	1/200	Santa Cruz	WB
Na/K-ATPase		Mouse	1/100	DSHB	WB
NF-200		Mouse	1/1.000	Sigma	IHC
NT-4		Rabbit	1/500	Merck Millipore	WB

$p75^{NTR}$		Goat	1/500	Merck Millipore	WB
PDK1		Mouse	1/200	Santa Cruz	WB
PDK1 (ser 241)		Rabbit	1/500	Cell Signalling	WB
cPKCα		Rabbit	1/800	Santa Cruz	WB
PKCα (Ser657)		Rabbit	1/1.000	Merck Millipore	WB
cPKCβI		Rabbit	1/800	Santa Cruz	WB/IHC
cPKCβI (thr 642)		Rabbit	1/500	Abcam	WB
S-100		Mouse/Rabbit	1/1.000	Acris antibodies/ Dako	IHC
Syntaxin		Mouse/ Rabbit	1/1.000	Sigma/ Cell Signalling	IHC
TrkB		Rabbit	1/200	Santa Cruz	WB
TrkB (tyr 816)		Rabbit	1/500	Merck Millipore	WB
IgG/Goat	HRP	Rabbit	1/10.000	Molecular probes	WB
IgG/Mouse	Alexa 488/647	Donkey	1/400	Molecular probes	IHC
IgG/Mouse	HRP	Rabbit	1/10.000	Sigma	WB
IgG/Rabbit	Alexa 488/647	Donkey	1/400	Molecular Probes	IHC
IgG/Rabbit	HRP	Donkey	1/10.000	J. Immunoresearch	WB

DSHB: Developmental Studies Hybridoma Bank

Reagents

Table 2 provides a list of reagents used in the different experimental procedures.

Reagent	Source	Working Solution	Function
μ-CgTx-GIIIB	Alomone	1.5 μM	To inhibit sarcolemmal voltage-dependent sodium channels (VDSCs) without affecting synaptic ACh release (Favreau et al., 1999)
TrkB antibody clone 47/TrkB	BD Transduction Laboratories	10 μg/ml	To inhibit the extracellular subdomains IgC1 and IgC2 of TrkB.
h-BDNF	Alomone	10mM	To mimic the effect of endogenous BDNF.
Tyrosine phosphatase inhibitor	Sigma	100-fold dilution	To ensure protection of TrkB from dephosphorylation.
βIV5-3 inhibitor peptide	Kindly provided by Dr. Mochly-Rosen from Stanford University	10 μM	To disrupt the interaction between cPKCβI and its specific βI-RACK. This inhibits its translocation to the membrane and its activation.

Presynaptic electrical stimulation of muscles

To study separately the effect of synaptic transmission from the effect of the muscle cell contraction in Chapter I, we designed 3 main experiments as show table 3.

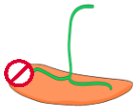
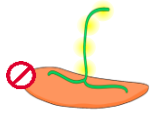
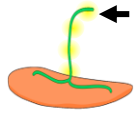
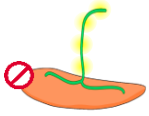
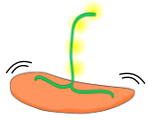
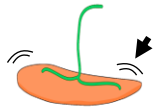
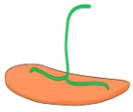
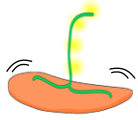
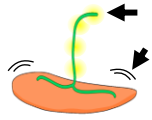
Exp.	Control treatment	Treatment	Outcome
#1	**No stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIIB preincubation. 3. Incubation in Ringer solution without stimulation. 	**Stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIIB preincubation. 3. Phrenic nerve stimulation with contraction blocked. 	**Effect of presynaptic stimulation** 
#2	**Stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIIB preincubation. 3. Phrenic nerve stimulation with contraction blocked. 	**Stimulation, contraction** 1. Hemidiaphragm extraction. 2. Preincubation in Ringer solution. 3. Phrenic nerve stimulation with contraction. 	**Effect of muscle contraction** 
#3	**No stimulation, not blocked contraction** 1. Hemidiaphragm extraction. 2. Incubation in Ringer solution without stimulation. 	**Stimulation, contraction** 1. Hemidiaphragm extraction. 2. Phrenic nerve stimulation with contraction. 	**Effect of presynaptic stimulation with contraction.** 

Table 3. Summary of the electrical stimulation experiments applied to extracted rat diaphragms.

Diaphragm muscle was excised together with its nerve supply and was placed in oxygenated Ringer solution (in nM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 12 and glucose 12.1mM) continuously bubbled with 95% O_2/5% CO_2 at room temperature. Finally, it was dissected into two hemidiaphragms, one for the treatment condition and the other one was used as its control. The phrenic nerve was stimulated *ex vivo* at 1 Hz by an A-M Systems 2100 isolated pulse generator (A-

M System, Carlsborg, WA). The frequency of 1 Hz allows the maintenance of different tonic functions (e.g., PKC activation) without promoting synaptic plasticity (e.g., facilitation). To separate the effect of synaptic transmission from the effect of the muscle cell contraction, we performed experiments in which contractions were prevented using μ-CgTx-GIIIB or not. Visible contractions of the diaphragm muscle served to verify successful nerve stimulation. Phrenic nerves were stimulated for 30 minutes unless otherwise noted. In all the cases, a minimum of 5 animals were used. The experimental design of the treatments is shown in table 3.

In Experiment **#1**, synaptic activity was assessed comparing presynaptically stimulated muscles blocked by μ-CgTx-GIIIB with non-stimulated muscles also incubated with μ-CgTx-GIIIB, to control for nonspecific effects of the blocker. In Experiment **#2**, muscle contraction per se was determined comparing stimulated/contracting muscles with stimulated muscles for which contraction was blocked. In Experiment **#3**, the effect of complete synaptic activity with resulting muscle contraction was assessed comparing stimulated/contracting muscles with non-stimulated muscles.

Training protocol

To study the effects of exercise in ALS mice in Chapter II, at 70 days of age, different training protocols were carry out for 30 minutes a day, 5 days a week as performed in previous studies (Gronard et al., 2008). Training was performed until 115 days of age or until death (Figure 14).

Swimming training: this training is associated with high frequency and amplitude hindlimb movements that preferentially activate a sub-population of fast motoneurons (with soma area greater than 700 μm2). Moreover, in plantaris muscle of ALS mice, this training efficiently prevents the fast-to-slow transition of fibres types that occurs during the disease, preserving the ALS muscle phenotype close to the controls muscles (Deforges et al., 2009). In this study, animals were

submitted to a swimming-based training program in an adjustable-flow swimming pool. The mice swam in a narrow lane, against an adjustable current of water. This swimming pool was specifically designed to train mice for an extended period of time. The mice were forced to swim due to the need to keep the head above water for breathing. They could not stop moving for more than a few seconds (Charbonnier and Soude, 2006, Patent FR 06 53772). The water flow was increased during 4 days up to 4 L min^{-1}. These trained animals are referred from now as Swimming ALS mice.

Running training: this training is associated with low hindlimb movement amplitude and frequency exercise, preferentially activating a sub-population of slow motoneurons (with soma area less than 300 µm2). In plantaris muscle, this training is associated with a fast-to-slow transition of fibres types, worsening the effects of the disease in ALS mice (Deforges et al., 2009). Animals were submitted to a moderate running-based training on a speed regulated treadmill. During of 4 days the speed was progressively increased to 13m min^{-1}. These trained animals are referred from now as Running ALS mice.

A third and fourth group of ALS and control mice (Untrained ALS and Control mice) were studied in parallel and only displayed an exploratory activity for the whole duration of training of the previously described animal groups. Once the training was finished, plantaris muscles were carefully dissected and frozen in liquid nitrogen. Then, the muscles were sent from Paris Descartes University (France) to our laboratory to perform Western Blot technique.

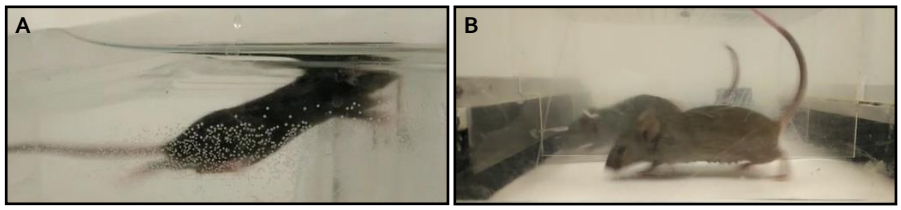


Figure 14. **Different training protocols applied**. **(A)** Swimming-based training, a high-frequency and -amplitude exercise. **(B)** Moderate running-based training, a low-frequency and -amplitude exercise.

Sample processing

Total lysate

Following standard protocols of stimulation, diaphragm muscles with the phrenic nerve were dissected, frozen in liquid nitrogen and stored at −80°C before use. Moreover, after receiving plantaris muscles from Paris Descartes University, samples were kept at -80°C until processed. They were both muscles homogenized using a high-speed homogenizer (overhead stirrer, VWR International, Clarksburg, MD) in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail 1% (Sigma-Aldrich, Saint Louis, MO, USA). Protein lysates were obtained collecting supernatants after removing insoluble materials by centrifugation at 1000g for 10 minutes at 4°C and then at 15000g for 20 minutes at 4°C. Protein concentrations were determined by DC protein assay (Bio-Rad, Hercules, CA, IL). Diaphragm samples were used to perform Western Blot and BDNF ELISA assay in Chapter I and plantaris samples to carry out Western Blot in Chapter II.

Membrane and cytosolic fractionation

To isolate the membrane and cytosolic fractions, after stimulation protocols, dissected diaphragm muscles were directly homogenized in ice-cold lysis buffer without detergents (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1 and sodium orthovanadate 1 and protease inhibitor cocktail (1/100)). The homogenized samples were cleared with a centrifugation at 1.000g at 4°C for 10 minutes, and the resulting supernatant was further centrifuged at 130.000g at 4°C for 1 hour. The supernatant corresponded to the cytosolic fraction and the pellet, to the membrane fraction. The latter was resuspended in lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail 1%). Validation of the purity of the subcellular fractionation was determined by examining the presence of fraction-specific housekeeping proteins like GAPDH for cytosol and Na/K-ATPase

for membrane by Western blot. Protein concentrations were determined as previously described. Samples were used to perform Western Blot in Chapter I.

BDNF ELISA assay

The BDNF Emax ImmunoAssay System (Promega Cat# G7610) was used to measure the amount of total BDNF (pro- and mature) in total lysates of stimulated diaphragm muscles. The protocol was performed according to the manufacturer's instructions and using a Sunrise Tecan A-5082 microplate reader set to 450 nm. Data was analysed with Magellan software (Tecan Group Ltd.) and the amount of detected BDNF was normalized to a standard curve and to the total protein content determined with the colorimetric assay (Bio-Rad).

Western Blot

To study BDNF/TrkB/PKC signalling pathway at the protein level, as well as PDK1, we performed Western Blot analysis. Samples of 15 or 30 µg were separated by 8% or 15% SDS-polyacrylamide electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Hybond™-P; Amersham, GE Healthcare). The membranes were blocked in TBST containing 5% (W/V) nonfat dry milk or 5% bovine serum albumin (BSA) and probed with the primary antibody overnight at 4°C (Table 1 describes the information of each antibody used). The membranes were incubated with the secondary antibody and visualized by chemiluminescence with the ECL kit (Amersham Life Science, Arlington Heights, IL). As a control, pre-treatment of a primary antibody with the appropriate blocking peptide (between three- and eightfold by weight) in skeletal muscle tissue was used to confirm its specificity preventing the immunolabelling.

The blots were imaged with the ChemiDoc XRS+Imaging System (Bio-Rad, Hercules, CA). The densitometry of the resultant bands was analysed with the ImageJ software (ImageJ). GAPDH and Na/K-ATPase proteins were used as loading

controls, as well as total protein staining (Sypro Ruby protein blot stain, Invitrogen). The integrated optical density of the bands was normalized with respect to (1) the background values and to (2) the total protein transferred on PVDF membranes, measured by total protein analysis (Aldridge et al., 2008). Specific phosphorylation was determined as the ratio of phosphorylated protein to total protein content. The relative variations between the experimental samples and the control samples were calculated from the same membrane image. The data were taken from densitometry measurements made in at least five separate experiments, plotted against controls.

Gene expression analysis

Gene expression analysis was done in collaboration with Michigan State University (United States). To determine changes in BDNF mRNA levels, four BDNF exons (IV, VI, VIII and IX) were studied by qRT-PCR. After 30 minutes of treatment (Table 3), diaphragm samples were frozen in RNase-free tubes in liquid nitrogen, sent to Michigan State University (United States) and held at -80°C until processed. Instruments used for dissection were cleaned with RNaseZap (Sigma-Aldrich) between animal harvests. RNeasy Fibrous Tissue Mini Kit (Qiagen) was used to extract RNA from muscle samples. Tissue was mechanically homogenized with a PRO200 homogenizer (Pro Scientific). Following extraction, RNA was quantified on a spectrophotometer (Beckman DU 530) by measuring 260 nm absorbance values. Extracted RNA was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with the following thermocycle: 25°C for 10 minutes, 37°C for 2 hours and 85°C for 5 minutes. Each qRT-PCR sample included 2.5 ng of cDNA, primers, and Power SYBR Green PCR Master Mix (Applied Biosystems). Thermocycle for the quantitative step on the ABI PRISM 7000 Sequence Detection System was as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A dissociation curve was determined for each well to confirm that only one product was amplified. Each sample was run in triplicate. Samples without reverse transcriptase during the

cDNA conversion were also assessed to ensure that there was no DNA contamination. The reference gene was 18S (400nM primers: GGAGCCTGCGGCTTAATTTG and CCACCCACGGAATCGAGAAA). In each experiment, we confirmed that levels of the reference gene were equivalent between treatment groups. Transcripts of four BDNF exons were quantified: IV (activity-dependent; 200nM primers: ACTGAAGGCGTGCGAGTATT and GGTGGCCGATATGTACTCCTG), VI (activity-dependent; 200nM primers: TCGCACGGTCCCCATTG and GGTCTCATCAAAGCCTGCCA), VIII (activity-independent; 400nM primers: AAACAAATTCTGCCAGTCCTGC and TTGGATAACTGCTCTGCTCCG) and IX (total transcripts; 200nM primers: GTCAAGTGCCTTTGGAGCCT and TGTTTGCGGCATCCAGGTAA). Optimal concentrations and amplification efficiencies were calculated for each primer set.

Immunohistochemistry and confocal microscopy

Diaphragm and LAL muscles were processed by IHC to detect and localize cPKCβI and PDK1 at the NMJ. There are target proteins commonly used to detect NMJ components: nerve by neurofilament or syntaxin, Schwann cell by S-100 and nAChR by α-bungarotoxin conjugated with tetramethylrhodamine (TRICT) (Figure 15). The information of each antibody and toxin is described in the table above (Table 1).

Whole muscle mounts were fixed with 4% paraformaldehyde for 30 minutes. After fixation, the muscles were rinsed with PBS and incubated in 0.1 M glycine in phosphate buffer saline (PBS). The muscles were permeabilized with 0.5-1% Triton X-100 in PBS, and nonspecific binding was blocked with 4% BSA. Then, muscles were incubated overnight at 4°C in mixtures of primary antibodies raised in different species and then rinsed. The muscles were then incubated for 4 hours at room temperature in a mixture of appropriate secondary antibodies. The secondary antibody specificity was tested by incubation in the absence of primary antibody. In multiple-staining protocols, omitting either one of the two primary

antibodies completely abolished the corresponding staining and there was no cross-reaction with the other primary antibodies. At least three muscles were used as negative controls.

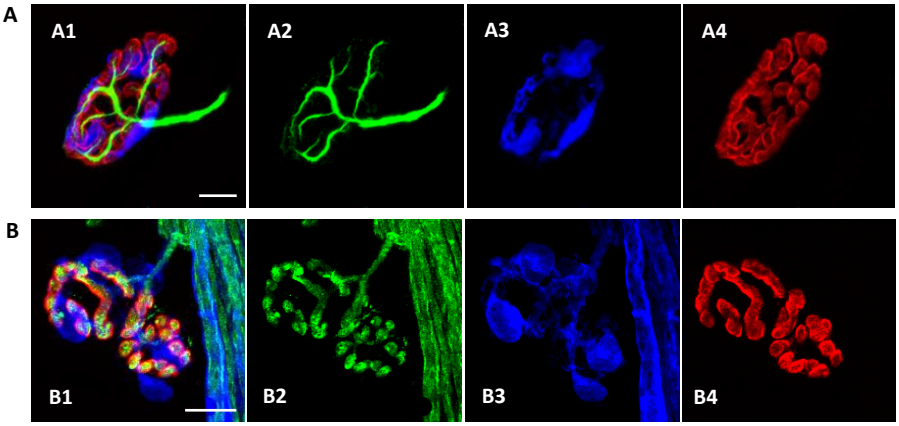


Figure 15. Example of neuromuscular junction (NMJ) labelled. (A) The conventional IHC labelling the nerve terminal with neurofilament. **(A1)** Merge of neurofilament-200, S-100 and α-bungarotoxin (α-BTX). **(A2)** Nerve terminal labelled with neuroflament-200 (in green). **(A3)** Schwann cell labelled with S-100 (in blue) and **(A4)** nAChRs labelled with α-BTX (in red). **(B)** The conventional IHC labelling the nerve terminal with syntaxin. **(B1)** Merge of syntaxin, S-100 and α-bungarotoxin (α-BTX). **(B2)** Nerve terminal labelled with syntaxin (in green) **(B3)** Schwann cell labelled with S-100 (in blue) and **(B4)** nAChRs labelled with α-BTX (in red). Scale bar = 10 µm.

For improved localization of the cPKCβI isoform at the NMJ, the muscles were processed to obtain semithin cross-sections from whole-mount multiple-immunofluorescent stained muscles. This method provided a simple and sensitive procedure for analysing the cellular distribution of molecules at the NMJ (Lanuza et al., 2007). After conventional IHC, the muscles were visualized in an inverted fluorescent microscope and the innervated areas were selected to obtain several samples of the muscle containing multiple NMJs. After dehydration with increasing concentrations of ethanol and acetone, the tissue fragments were embedded in Spurr's resin (plastic) in transverse orientation. Sections 0.5–0.7 µm thick were cut with a Reichert Ultracut E microtome (Leica Microsystems, Bannockburn, IL) and

flattened on glass slides by heating on a hotplate. The fluorescent serial sections were mounted with paraphenyldiamine and mowiol to retard fading.

Immunolabelled NMJs from the whole-mount muscles and the semithin cross-sections were viewed with a laser-scanning confocal microscope (Nikon TE2000-E). Special consideration was given to the possible contamination of one channel by another. In experiments involving negative controls, the photomultiplier tube gains and black levels were identical to those used for a labelled preparation made in parallel with the control preparations. At least 25 endplates per muscle were observed, and at least five muscles were studied. Images were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA) and neither the contrast nor brightness were modified.

Electrophysiology

To study the involvement of cPKCβI in neurotransmission we performed electrophysiological techniques. Diaphragm muscles were removed surgically and incubated in a Sylgard-Petri dish containing normal Ringer solution (in mM): NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 15, glucose 11 continuously bubbled with 95% O_2 and 5% CO_2. Temperature and humidity were set to 26°C and 50%, respectively. Spontaneous mEPPs and EPPs were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (resistance: 20-40 MW). Recording electrodes were connected to an amplifier (Tecktronics, AMS02), and a distant Ag-AgCl electrode connected to the bath solution via an agar bridge (agar 3.5% in 137 mM NaCl) was used as a reference. The signals were digitized (DIGIDATA 1322A Interface, Axon Instruments Inc, CA, USA), stored and computer-analyzed with the software Axoscope 9.0 (Axon Instruments Inc, CA, USA).

Measures have to be taken to prevent muscle action potentials, since these obscure the EPP and cause contraction which in general will disrupt the recording. So, to prevent muscle contraction during EPP recordings, we used μ-CgTx-GIIIB (See

table 2). After a muscle fibre had been impaled, the phrenic nerve was continuously stimulated (70 stimuli, 1Hz) using two platinum electrodes that were coupled to a pulse generator (CIBERTEC CS-20) linked to a stimulus isolation unit. Thus, in stimulated muscles, we recorded and measured control EPPs and then, we incubated the muscle in βIV5-3 inhibitor peptide for one hour (to block cPKCβI activation). The last 50 EPPs were recorded. We selected fibres with membrane potentials of no less than -70mV and used only those results from preparations which did not deviate by more than 5mV during the recording. The mean amplitude (mV) per fibre was calculated and corrected for non-linear summation (EPPs were usually more than 4 mV) (McLachlan and Martin, 1981) assuming a membrane potential of –80 mV. We studied a minimum of 15 fibres per muscle and usually a minimum of 5 muscles in each type of experiment.

Statistical analysis

Values are expressed as means ± SEM. The statistical software SPSS© v17.0 (SPSS) was used to analyse the results. The normality of the distributions was tested with the Kolmogorov–Smirnov test or Shapiro-Wilk. Statistical significance of the differences between groups was evaluated under the Wilcoxon test or the Student's t-test. For electrophysiology techniques, we used the two-tailed Welch's t-test. Statistical significance of results in Chapter II was evaluated under 2-way ANOVA.

Relative Expression Software Tool (REST) was used to assess statistical significance and fold change of genes in gene expression analysis (Pfaffl et al., 2002). Specifically, this software uses the non-parametric Pair-Wise Fixed Reallocation Randomization Test.

The criterion for statistical significance was ***$P < 0.001$, **$0.001 < P < 0.01$, *$0.01 < P < 0.05$.

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D. RESULTS

Chapter I

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RESULTS

PUBLICATION 1:

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1Unitat d'Histologia i Neurobiologia Universitat Rovira i Virgili, Reus, Spain.
2Neuroscience Program, Michigan State University, Michigan, MI, United States.

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Muscle Contraction Regulates BDNF/TrkB Signaling to Modulate Synaptic Function through Presynaptic cPKCα and cPKCβI

Erica Hurtado[1], Víctor Cilleros[1], Laura Nadal[1], Anna Simó[1], Teresa Obis[1], Neus Garcia[1†], Manel M. Santafé[1], Marta Tomàs[1], Katherine Halievski[2], Cynthia L. Jordan[2], Maria A. Lanuza[1†] and Josep Tomàs[1*†]*

[1]Unitat d'Histologia i Neurobiologia (UHNEUROB), Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain, [2]Neuroscience Program, Michigan State University, Michigan, MI, United States

The neurotrophin brain-derived neurotrophic factor (BDNF) acts via tropomyosin-related kinase B receptor (TrkB) to regulate synapse maintenance and function in the neuromuscular system. The potentiation of acetylcholine (ACh) release by BDNF requires TrkB phosphorylation and Protein Kinase C (PKC) activation. BDNF is secreted in an activity-dependent manner but it is not known if pre- and/or postsynaptic activities enhance BDNF expression *in vivo* at the neuromuscular junction (NMJ). Here, we investigated whether nerve and muscle cell activities regulate presynaptic conventional PKC (cPKCα and βI) via BDNF/TrkB signaling to modulate synaptic strength at the NMJ. To differentiate the effects of presynaptic activity from that of muscle contraction, we stimulated the phrenic nerve of rat diaphragms (1 Hz, 30 min) with or without contraction (abolished by μ-conotoxin GIIIB). Then, we performed ELISA, Western blotting, qRT-PCR, immunofluorescence and electrophysiological techniques. We found that nerve-induced muscle contraction: (1) increases the levels of mature BDNF protein without affecting pro-BDNF protein or BDNF mRNA levels; (2) downregulates TrkB.T1 without affecting TrkB.FL or p75 neurotrophin receptor (p75) levels; (3) increases presynaptic cPKCα and cPKCβI protein level through TrkB signaling; and (4) enhances phosphorylation of cPKCα and cPKCβI. Furthermore, we demonstrate that cPKCβI, which is exclusively located in the motor nerve terminals, increases activity-induced acetylcholine release. Together, these results show that nerve-induced muscle contraction is a key regulator of BDNF/TrkB signaling pathway, retrogradely activating presynaptic cPKC isoforms (in particular cPKCβI) to modulate synaptic function.

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***Correspondence:**
Maria A. Lanuza
mariaangel.lanuza@urv.cat
Josep Tomàs
josepmaria.tomas@urv.cat

†These authors have contributed equally to this work.

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Abbreviations: ACh, Acetylcholine; AChRs, acetylcholine receptors; BSA, bovine serum albumin; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; cPKC, conventional protein kinase C; cPKC cPKCα, conventional protein kinase C alpha; cPKC βI, conventional protein kinase C beta I; βIV5–3, cPKCβI-specific translocation inhibitor peptide; EPPs, evoked endplate potentials; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; LAL, levator auris longus; MAPK, mitogen-activated protein kinase; MARCKS, myristoylated alanine-rich C-kinase substrate; NMJ, neuromuscular junction; NT-4, neurotrophin-4; p75, p75 neurotrophin receptor; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate buffer saline; PLC, phospholipase C; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RACK, receptor for activated C-kinase; MEPPs, spontaneous miniature endplate potentials; TSBT, tris-buffered saline, Tween 20; TrkB, tropomyosin receptor kinase B; TrkB.FL, tropomyosin receptor kinase B full length isoform; TrkB.T1, tropomyosin receptor kinase B truncated isoform 1; TrkB.T2, tropomyosin receptor kinase B truncated isoform 2; α-BTX, α-bungarotoxin; μ-CgTx-GIIIB, μ-conotoxin GIIIB.

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These results indicate that a decrease in neuromuscular activity, as occurs in several neuromuscular disorders, could affect the BDNF/TrkB/PKC pathway that links pre- and postsynaptic activity to maintain neuromuscular function.

Keywords: Bdnf-TrkB signaling, neuromuscular junction, PKC, muscle contraction, neurotransmission

INTRODUCTION

Nerves and skeletal muscles interact via two modes of communication: electrical activity and neurotrophic regulation (Baldwin et al., 2013; Cisterna et al., 2014). Nerve impulses generated in the central nervous system (CNS) trigger muscle contraction via electromechanical coupling. On the other hand, neurotrophic control acts via the release of neurotrophic factors, including the neurotrophins, and regulates the development, differentiation, survival and function of the nerve terminal (Wang et al., 1995; Mantilla et al., 2004). One of the most studied neurotrophins is brain-derived neurotrophic factor (BDNF; Hofer and Barde, 1988; Barde, 1990; Bibel and Barde, 2000). BDNF is initially synthesized as a precursor (proBDNF), which is cleaved to a mature isoform (mBDNF) via intracellular or extracellular proteases. The two isoforms induce different and even opposite functions by binding preferentially to the low-affinity nerve growth factor receptor (p75) or the tropomyosin-related kinase B receptor (TrkB; Lu, 2003; Hempstead, 2006; Kermani and Hempstead, 2007; Yang et al., 2009; Je et al., 2013). In addition, alternative splicing of TrkB mRNA gives rise to a full-length TrkB isoform (TrkB.FL) and two truncated TrkB isoforms T1 and T2 (TrkB.T1 and TrkB.T2), which lack part of the intracellular kinase domain (Middlemas et al., 1991; Reichardt, 2006). TrkB.T1 is the main truncated isoform in the skeletal muscle, being TrkB.T2 a variant more predominant in the brain tissue (Stoilov et al., 2002). Evidence suggests that heterodimers of TrkB.FL with the truncated isoforms inhibit trans-autophosphorylation of TrkB.FL, reduce BDNF signaling or even may signal independently (Eide et al., 1996; Baxter et al., 1997; Rose et al., 2003; Dorsey et al., 2012; Wong and Garner, 2012).

Increasing evidence suggests that exercise training benefits CNS health, including the improvement of synaptic function (van Praag et al., 1999). BDNF seems to play a key role in mediating the benefits of exercise (Neeper et al., 1995; Vaynman et al., 2006; Gomez-Pinilla et al., 2008; Zoladz and Pilc, 2010; Gomez-Pinilla and Hillman, 2013). In particular, BDNF is secreted in an activity-dependent manner (Lu, 2003) and its expression in rodent spinal cord and skeletal muscle increases after exercise (Gómez-Pinilla et al., 2001, 2002; Cuppini et al., 2007; Gomez-Pinilla et al., 2012). Likewise, basal levels of neuromuscular activity are required to maintain normal levels of BDNF in the neuromuscular system (Gómez-Pinilla et al., 2002). Recently, it was shown that cultured myotubes release BDNF when stimulated to contract, suggesting a postsynaptic origin of this neurotrophin (Matthews et al., 2009). Unfortunately, whether skeletal muscles *in vivo* increase their production and/or release of BDNF by synaptic activity, muscle contraction or some combination of the two, remains unclear. Furthermore, exogenous BDNF increases evoked acetylcholine (ACh) release at the neuromuscular junction (NMJ) and the TrkB receptor is normally coupled to this process (Knipper et al., 1994; Mantilla et al., 2004; Garcia et al., 2010; Santafé et al., 2014). Together, this and other findings support the idea that neuromuscular activity promotes BDNF/TrkB retrograde signaling to regulate neuromuscular function (Kulakowski et al., 2011; Dorsey et al., 2012), an idea we now test.

The potentiation of presynaptic vesicle released by BDNF requires TrkB phosphorylation and phospholipase C (PLC) activation (Middlemas et al., 1994; Kleiman et al., 2000). In turn, PLCγ activates Protein Kinase C (PKC) which interacts with TrkB to modulate neurotransmission at the NMJ (West et al., 1991; Numann et al., 1994; Byrne and Kandel, 1996; Catterall, 1999; Santafé et al., 2005, 2006, 2014). In the NMJ, synaptic activity depends on the influx of calcium, and presynaptic calcium-dependent PKC (cPKC) isoforms have been shown to modulate neurotransmission (Santafé et al., 2005, 2006; Besalduch et al., 2010). However, which cPKC isoforms are involved in ACh release remains unknown. The cPKCβI and cPKCα isoforms are good candidates because of their presynaptic location, with PKCβI being present exclusively in the nerve terminal of the NMJ (Besalduch et al., 2010). Recently, we have shown that muscle contraction *per se* enhances the levels of presynaptic PKC isoforms (α, βI and ε; Besalduch et al., 2010; Obis et al., 2015a). This suggests that a retrograde signal induced by muscle contractile activity can regulate presynaptic PKC isoforms.

Here, we investigated the hypothesis that nerve-induced muscle activity regulates BDNF/TrkB signaling pathway to modulate synaptic function via activation of presynaptic cPKC isoforms.

MATERIALS AND METHODS

Animals

Diaphragm and levator auris longus (LAL) muscles were obtained from Sprague-Dawley rats (30–40 days; Criffa, Barcelona, Spain). The animals were cared for in accordance with the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All the procedures realized were reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili (URV; Reference number: 0289). At least five independent animals ($n > 5$) were used to evaluate the following techniques.

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Antibodies

Primary antibodies used for Western blot were rabbit anti-BDNF (Cat# sc-20981), rabbit anti-PKCα (Cat# sc-208), rabbit anti-PKCβI (Cat# sc-209), rabbit anti-TrkB (Cat# sc-8316) and goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cat# sc-20358) polyclonal antibodies, purchased from Santa Cruz Biotechnology. Rabbit anti-pPKCα (ser657; Cat# 07-790), goat anti-p75 (Cat# AB1554), rabbit anti-neurotrophin-4 (NT-4; Cat# AB1781SP) and rabbit anti-pTrkB (tyr816; Cat# AB1381) antibodies were purchased from Merck Millipore. Rabbit anti-pPKCβI (thr 641; Cat# ab75657) polyclonal antibody was purchased from Abcam. The secondary antibodies used were donkey anti-rabbit conjugated to horseradish peroxidase (HRP) from Jackson Immunoresearch Labs (Cat# 711-035-152) and rabbit anti-goat conjugated to HRP from Molecular probes (Cat# R21459). Immunohistochemistry was performed with antibodies that are commonly used as markers to differentially detect the components of the NMJ (syntaxin, neurofilament-200 and S100): mouse anti-syntaxin (Cat# S0664) and mouse anti-neurofilament-200 (Cat# N2912) monoclonal antibodies were purchased from Sigma. Mouse anti-S100 monoclonal antibody (Cat# AM10036FC-N) was from Acris Antibodies. Rabbit anti-PKCβI polyclonal antibody was purchased from Santa Cruz Biotechnology (Cat# sc-209). The secondary antibodies used were donkey anti-rabbit or anti-mouse conjugated to Alexa Fluor 488 and Alexa Fluor 647 from Molecular Probes (Eugene, OR, USA; Cat# A21206; Cat# A21202; Cat# A-31573; Cat# A-31571). Postsynaptic AChRs were detected with α-bungarotoxin (α-BTX) conjugated to tetramethylrhodamine (TRITC) from Molecular Probes (Eugene, OR, USA; Cat# T1175). As a control, primary antibodies were omitted from some muscles during the immunohistochemical and Western blot procedures. These control muscles never exhibited positive staining or revealed bands of the appropriate molecular weight with the respective procedures. In double-staining protocols, omitting either one of the two primary antibodies completely abolished the corresponding staining and there was no cross-reaction with the other primary antibody. Pretreatment of a primary antibody with the appropriate blocking peptide (between three- and eight-fold by weight) in skeletal muscle tissue prevented immunolabelling.

Reagents

In presynaptic stimulation treatments and electrophysiological experiments, muscle contraction was blocked using μ-conotoxin GIIIB (μ-CgTx-GIIIB, Alomone Labs Ltd, Jerusalem, Israel). This toxin selectively inhibits sarcolemmal voltage-dependent sodium channels (VDSCs) without affecting synaptic ACh release (Favreau et al., 1999). It was supplied as lyophilized powder of >99% purity. The working concentration was 1.5 μM in Ringer's solution (see below).

The anti-TrkB antibody clone 47/TrkB (BD Transduction Laboratories Cat# 610101) was used for TrkB inhibition assays. The working solution was 10 μg/ml. For BDNF exogenous incubations we used h-BDNF (Alomone Labs; Cat# B-250) 10 mM.

Phosphatase inhibition experiments were performed using a phosphatase inhibitor cocktail from Sigma-Aldrich (St. Louis, MO, USA) in a 100-fold dilution.

To block cPKCβI activity we used the specific translocation inhibitor peptide βIV5–3 at 10 μM (kindly provided by Dr. Mochly-Rosen from Stanford University). It is derived from the V5 domain of cPKCβI and binds to the anchoring protein RACK (receptors for activated C-kinase), disrupting the interaction between cPKCβI and its specific βI-RACK. This inhibits its translocation to the membrane and its activation.

Presynaptic Electrical Stimulation of Muscles

Diaphragm muscle—a typical model to study the development and function of the NMJ (Yang et al., 2001; Li et al., 2008; Wu et al., 2010)—was excised together with its nerve supply and dissected into two hemidiaphragms. One hemidiaphragm underwent a treatment and the other one was used as its control. The experimental design of the treatments is shown in **Table 1**. The protocol of electrical stimulation followed that of Besalduch et al. (2010) and Obis et al. (2015a). Briefly, each hemidiaphragm muscle with the phrenic nerve was placed in oxygenated Ringer solution (in nM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 12 and glucose 12.1 mM) continuously bubbled with 95% O_2/5% CO_2 at room temperature. The phrenic nerve was stimulated *ex vivo* at 1 Hz by an A-M Systems 2100 isolated pulse generator (A-M System, Carlsborg, WA, USA). The frequency of 1 Hz allows the maintenance of different tonic functions (e.g., PKC activation) without promoting synaptic plasticity (e.g., facilitation). To study separately the effect of synaptic transmission from the effect of the muscle cell contraction, we performed experiments in which contractions were prevented using μ-CgTx-GIIIB or not. Visible contractions of the diaphragm muscle served to verify successful nerve stimulation. Three main experiments were performed to discern the effects of synaptic activity from that of muscle activity (**Table 1**). Each experiment involved a specific treatment and its control. In Experiment #1, synaptic activity was assessed comparing presynaptically stimulated muscles blocked by μ-CgTx-GIIIB with non-stimulated muscles also incubated with μ-CgTx-GIIIB to control for nonspecific effects of the blocker. In Experiment #2, muscle contraction *per se* was determined comparing stimulated/contracting muscles with stimulated muscles for which contraction was blocked. In Experiment #3, the effect of complete synaptic activity with resulting muscle contraction was assessed comparing stimulated/contracting muscles with non-stimulated muscles, without exposure to μ-CgTx-GIIIB. Phrenic nerves were stimulated for 30 min unless otherwise noted. In all the cases, a minimum of five animals were used.

BDNF ELISA Assay

Diaphragm muscles were dissected, frozen in liquid nitrogen and homogenized using a high-speed homogenizer (overhead stirrer, VWR International, Clarksburg, MD, USA) in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50,

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TABLE 1 | Summary of the electrical stimulation experiments applied to extracted rat diaphragms.

Experiment	Control treatment	Treatment	Final outcome
#1 Presynaptic stimulation	**No stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIB preincubation. 3. Incubation in Ringer solution without stimulation.	**Stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIB preincubation. 3. Phrenic nerve stimulation with contraction blocked.	**Effect of presynaptic stimulation**
#2 Contraction	**Stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIB preincubation. 3. Phrenic nerve stimulation with contraction blocked.	**Stimulation, contraction** 1. Hemidiaphragm extraction. 2. Preincubation in Ringer solution. 3. Phrenic nerve stimulation with contraction.	**Effect of muscle contraction**
#3 Presynaptic stimulation with contraction	**No stimulation, not blocked contraction** 1. Hemidiaphragm extraction. 2. Incubation in Ringer solution without stimulation.	**Stimulation, contraction** 1. Hemidiaphragm extraction. 2. Phrenic nerve stimulation with contraction.	**Effect of presynaptic stimulation with contraction.**

Three main experiments were designed in order to separately study the effect of the presynaptic stimulation (and synaptic transmission), the effect of muscle cell contraction and the effect of presynaptic stimulation with muscle contraction. Each diaphragm was divided into two hemidiaphragms, one underwent a treatment and the other served as its particular control.

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PMSF 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail 1% (Sigma-Aldrich, St. Louis, MO, USA). Protein lysates were obtained collecting supernatants after removing insoluble materials by centrifugation at 1000 *g* for 10 min at 4°C and then at 15,000 *g* for 20 min at 4°C.

Protein concentrations were determined by DC protein assay (Bio-Rad, Hercules, CA, USA). The BDNF Emax ImmunoAssay System (Promega Cat# G7610) was used to measure the amount of total BDNF (pro- and mature) in each sample following standard protocols. Plates were coated with a specific anti-BDNF monoclonal antibody and then washed once using tris-buffered saline, 0.1% Tween 20 (TSBT). Plates were blocked using 200 μL Promega 1× Block and Sample buffer. After washing, diaphragm samples and BDNF protein standards (0–500 pg BDNF protein) were added in triplicate to the plates and incubated for 2 h at room temperature. Plates were washed five times and anti-human BDNF polyclonal antibody was added to each well. Plates were washed again five times using TBST wash buffer. The sandwich was completed by adding anti-IgY HRP conjugate. Finally, plates were developed using stabilized chromogen (tetramethylbenzidine) and the reaction was stopped using 1 N HCl. Absorbance was read at 450 nm using a Sunrise Tecan A-5082 microplate reader and data was analyzed with Magellan software (Tecan Group Ltd.). Afterwards, the amount of detected BDNF was normalized to a standard curve and to the total protein content determined with the colorimetric assay (Bio-Rad).

Western Blot

Diaphragm muscles with the phrenic nerve were dissected, frozen in liquid nitrogen, and stored at −80°C before use. Homogenization, lysate obtainment and determination of protein concentration were performed with the same protocols and solutions described previously for BDNF ELISA assay. The linear and quantitative dynamic range for each target protein and the appropriate dilutions of samples were determined for accurate and normalized quantitation of densitometric analysis.

To isolate the membrane and cytosolic fractions, diaphragm muscles were dissected and homogenized in ice-cold lysis buffer without detergents (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1 and sodium orthovanadate 1 and protease inhibitor cocktail (1/100). The homogenized samples were cleared with a centrifugation at 1000 *g* at 4°C for 15 min, and the resulting supernatant was further centrifuged at 130,000 *g* at 4°C for 1 h. The supernatant corresponded to the cytosolic fraction and the pellet, to the membrane fraction. The latter was resuspended in lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail 1% (Sigma-Aldrich, St. Louis, MO, USA)). Fractionation was assessed by blotting of GAPDH, a specific cytosolic protein. GADPH immunoreactivity was never observed in the membrane fraction. Protein concentrations were determined as previously described.

Protein samples of 15 μg or 30 μg were separated by 8% or 15% SDS-polyacrylamide electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Hybond^{TM}-P; Amersham, GE Healthcare). The membranes were blocked in TBST containing 5% (W/V) nonfat dry milk or 5% bovine serum albumin (BSA) and probed with the primary antibody overnight at 4°C. The membranes were incubated with the secondary antibody and visualized by chemiluminescence with the ECL kit (Amersham Life Science, Arlington Heights, IL, USA).

The blots were visualized with the ChemiDoc XRS+Imaging System (Bio-Rad, Hercules, CA, USA). The densitometry of the resultant bands was analyzed with the ImageJ software (ImageJ). The integrated optical density of the bands was normalized with respect to: (1) the background values; and to (2) the total protein transferred on PVDF membranes, measured by total protein analysis (Sypro Ruby protein blot stain, Bio-rad; Aldridge et al., 2008). In some cases, β-actin blotting and total protein staining were used as a loading controls resulting in the same normalization values. Specific phosphorylation was determined as the ratio of phosphorylated protein to total protein content. The relative variations between the experimental samples and the control samples were calculated from the same membrane image. The data were taken from densitometry measurements made in at least five separate experiments, plotted against controls. Data are mean values ± SEM. Statistical significance of the differences between groups was evaluated under the Wilcoxon test or the Student's *t*-test and the normality of the distributions was tested with the Kolmogorov–Smirnov test. The criterion for statistical significance was $p < 0.05$ vs. the control (*).

Gene Expression Analysis

Gene expression was analyzed via qRT-PCR in separate cohorts of rats from the three stimulation treatments (see **Table 1**) targeting four BDNF exons (IV, VI, VIII and IX), to determine whether changes in BDNF mRNA levels might underlie the changes in BDNF protein and whether this occurs preferentially at known activity-dependent exons.

Each rat provided one hemidiaphragm for the control group and the other hemidiaphragm for the experimental group (n = 6–7 hemidiaphragms per group), as described above for protein analysis. After 30 min of treatment (**Table 1**), tissue samples were frozen in RNase-free tubes in liquid nitrogen, and held at −80°C until processed. Instruments used for dissection were cleaned with RNaseZap (Sigma-Aldrich) between animal harvests.

RNeasy Fibrous Tissue Mini Kit (Qiagen) was used to extract RNA from muscle samples. Tissue was mechanically homogenized with a PRO200 homogenizer (Pro Scientific). Following extraction, RNA was quantified on a spectrophotometer (Beckman DU 530) by measuring 260 nm absorbance values. Extracted RNA was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with the following thermocycle: 25°C for 10 min, 37°C for 2 h and 85°C for 5 min. Each qRT-PCR sample included 2.5 ng of cDNA, primers, and Power SYBR Green PCR Master Mix (Applied Biosystems). Thermocycle for the quantitative step on the ABI PRISM 7000 Sequence Detection System was as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

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A dissociation curve was determined for each well to confirm that only one product was amplified. Each sample was run in triplicate. Samples without reverse transcriptase during the cDNA conversion were also assessed to ensure that there was no DNA contamination. The reference gene was 18S (400 nM primers: GGAGCCTGCGGCTTAATTTG and CCACCCACGGAATCGAGAAA). In each experiment, we confirmed that levels of the reference gene were equivalent between treatment groups. Transcripts of four BDNF exons were quantified: IV (activity-dependent; 200 nM primers: ACTGAAGGCGTGCGAGTATT and GGTGGCCGATATGTACTCCTG), VI (activity-dependent; 200 nM primers: TCGCACGGTCCCCATTG and GGT CTCATCAAAGCCTGCCA), VIII (activity-independent; 400 nM primers: AAACAAATTCTGCCAGTCCTGC and TTGGATAACTGCTCTGCTCCG) and IX (total transcripts; 200 nM primers: GTCAAGTGCCTTTGGAGCCT and TGTTTGCGGCATCCAGGTAA). Optimal concentrations and amplification efficiencies were calculated for each primer set.

Relative Expression Software Tool (REST) was used to assess statistical significance and fold change of genes (Pfaffl et al., 2002). Specifically, this software uses the non-parametric Pair-Wise Fixed Reallocation Randomization Test to account for amplification efficiencies when determining fold change. It measures relative expression of a target gene (BDNF transcripts IV, VI, VIII and total IX) between a control and sample group following the normalization of the target gene to a reference gene (18S). The criterion for statistical significance was $p < 0.05$ (*).

Immunohistochemistry and Confocal Microscopy

Diaphragm and LAL muscles were processed by immunohistochemistry to detect and localize the cPKCβI isoform at the NMJ. LAL muscle permits a better imaging and analysis of NMJs within the muscle. Whole muscle mounts were fixed with 4% paraformaldehyde for 30 min. After fixation, the muscles were rinsed with PBS and incubated in 0.1 M glycine in phosphate buffer saline (PBS). The muscles were permeabilized with 0.5% Triton X-100 in PBS, and nonspecific binding was blocked with 4% BSA. Then, muscles were incubated overnight at 4°C in mixtures of three primary antibodies raised in different species (anti cPKCβI isoform; anti-syntaxin and anti-neurofilament-200 to label the axon terminal; anti-S100 to label Schwann cells) and then rinsed. The muscles were then incubated for 4 h at room temperature in a mixture of appropriate secondary antibodies. Acetylcholine receptors (AChRs) were detected with α-BTX conjugated with TRITC. At least three muscles were used as negative controls as described above. No crossover was detected between antibodies. For improved localization of the cPKCβI isoform at the NMJ, the muscles were processed to obtain semithin cross-sections from whole-mount multiple-immunofluorescent stained muscles. This method provided a simple and sensitive procedure for analyzing the cellular distribution of molecules at the NMJ (Lanuza et al., 2007). Immunolabeled NMJs from the whole-mount muscles and the semithin cross-sections were viewed with a laser-scanning confocal microscope (Nikon TE2000-E). Special consideration was given to the possible contamination of one channel by another. In experiments involving negative controls, the photomultiplier tube gains and black levels were identical to those used for a labeled preparation made in parallel with the control preparations. At least 25 endplates per muscle were observed, and at least six muscles were studied. Images were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA) and neither the contrast nor brightness were modified.

Electrophysiology

Diaphragm muscles were removed surgically and incubated in a Sylgard-Petri dish containing normal Ringer solution (in mM): NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 15, glucose 11 continuously bubbled with 95% O_2 and 5% CO_2. Temperature and humidity were set to 26°C and 50%, respectively. Spontaneous miniature endplate potentials (MEPPs) and evoked EPPs (EPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (resistance: 20–40 MW). Recording electrodes were connected to an amplifier (Tecktronics, AMS02), and a distant Ag-AgCl electrode connected to the bath solution via an agar bridge (agar 3.5% in 137 mM NaCl) was used as a reference. The signals were digitized (DIGIDATA 1322A Interface, Axon Instruments Inc., Union City, CA, USA), stored and computer-analyzed. The software Axoscope 9.0 (Axon Instruments Inc., Union City, CA, USA) was used for data acquisition and analysis. To prevent muscle contraction during EPP recordings, we used μ-CgTx-GIIIB (1, 5 μM). After a muscle fiber had been impaled, the phrenic nerve was continuously stimulated (70 stimuli, 1 Hz) using two platinum electrodes that were coupled to a pulse generator (CIBERTEC CS-20) linked to a stimulus isolation unit. Thus, in stimulated muscles, we recorded and measured control EPPs and then, we incubated the muscle in βIV_{5-3} inhibitor peptide for 1 h. The last 50 EPPs were recorded. We selected fibers with membrane potentials of no less than −70 mV and used only those results from preparations which did not deviate by more than 5 mV during the recording. The mean amplitude (mV) per fiber was calculated and corrected for non-linear summation (EPPs were usually more than 4 mV; McLachlan and Martin, 1981) assuming a membrane potential of −80 mV. We studied a minimum of 15 fibers per muscle and usually a minimum of five muscles in each type of experiment. The statistical software SPSS© v17.0 (SPSS, RRID:SCR_002865) was used to analyze the results. Values are expressed as means ± SEM. Only one hemidiaphragm was used from each animal for a given experiment. We used the two-tailed Welch's *t*-test (for unpaired values and variances were not assumed to be equal). Differences were considered significant at $p < 0.05$ (*).

RESULTS

To determine the relationship between neuromuscular activity and neurotrophic control, we developed an *in vivo* experimental

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system in which we can distinguish the effects of synaptic activity from that of muscle contraction (see **Table 1**). Synaptic activity includes normal presynaptic stimulus, synaptic transmission and endplate potential generation due to ACh signaling. Muscle contraction includes membrane depolarization of the muscle fiber involving voltage-dependent sodium channels and the resulting myofiber contraction. Thus, the effects of synaptic activity were determined by comparing muscles that had contraction blocked (with μ-CgTx-GIIIB), but the nerve was stimulated in one case but not the other (referred to as the *Stimulation* condition in the figures). The effects of muscle contraction were determined by comparing muscles which were both stimulated via their nerves, but in one case muscle contraction was blocked (referred to as the *Contraction* condition in the figures). Finally, presynaptic *Stimulation with Contraction* treatment comprises the effects of synaptic activity and muscle contraction, showing complete neuromuscular activity.

Both Synaptic Activity and Muscle Contraction Enhance BDNF Protein Levels in the Skeletal Muscle

Neuromuscular activity (e.g., through physical exercise) increases BDNF expression in the skeletal muscle, CNS and plasma (Gómez-Pinilla et al., 2001, 2002; Cuppini et al., 2007; Zoladz and Pilc, 2010; Gomez-Pinilla et al., 2012). However, it has been assumed that enhanced BDNF levels is caused by muscle activity *per se*, but this has not been directly shown. Therefore, our first objective was to determine whether synaptic activity and/or muscle contraction regulate BDNF protein levels in the skeletal muscle. Thus, we compare BDNF protein levels in muscles stimulated via the nerve (1 Hz) for which contraction was blocked or not. Each hemidiaphragm was compared to a corresponding control from the same animal (see **Table 1**). BDNF levels were measured using ELISA and results showed that nerve stimulation significantly elevated BDNF levels, either in the presence of contraction or when it was blocked. Synaptic activity raised total BDNF levels in muscle by 72.8% ± 8.6 (**Figure 1A**). Likewise, muscle contraction further increased total BDNF levels by 38.9% ± 6.5 (**Figure 1A**). In concordance, combined synaptic and muscle activity increased BDNF levels by 104.0% ± 19.9, indicating that the effects of synaptic vs. muscle activity had additive effects in increasing significantly the level of total BDNF protein in muscle. That nerve activity without muscle contraction potently increases BDNF expression in muscle is a novel finding. Altogether, these results indicate that both synaptic activity and muscle contraction enhance BDNF protein levels in skeletal muscle. Moreover, muscle contraction *per se* increases the BDNF levels above the rise produced by synaptic activity without contraction, significantly contributing to the total protein amount in skeletal muscle.

Synaptic Activity and Muscle Contraction each Increase Mature BDNF Protein Levels

Although results of the ELISA allowed us to quantify accurately the total BDNF protein levels in muscle for each experiment, this technique cannot tell us how the mBDNF and pro-BDNF are modulated by activity. Because pro-BDNF and mBDNF produce different, and even opposite, effects (Lu, 2003; Woo et al., 2005; Hempstead, 2006; Yang et al., 2009; Je et al., 2013), we used Western blotting to analyze how activity affected the level of mBDNF and pro-BDNF separately. We used an anti-BDNF antibody raised against a peptide sequence corresponding to the amino acids 130–247 of BDNF, a region present in both pro-BDNF (32 kDa) and mBDNF (14 kDa; Zheng et al., 2010). BDNF Western blotting (**Figure 1B**) shows that both presynaptic stimulation and muscle contraction each significantly increased mBDNF protein levels without affecting pro-BDNF. In particular, presynaptic stimulation induced a 72.5% ± 12.4 ($p < 0.05$) increase in mBDNF levels with respect to basal conditions whereas muscle contraction further increased mBDNF levels by 45.2% ± 7.5 ($p < 0.05$). Although the source of this increased BDNF is not clear, our results show that activity in either the synapse or the muscle increase mature BDNF without affecting pro-BDNF protein levels in skeletal muscle.

That pro-BDNF levels were maintained in the face of increases in mBDNF levels suggests that synthesis of this isoform is tightly regulated, otherwise, pro-BDNF levels would decrease after the upregulation of its cleavage into mature BDNF. Thus, we examined gene expression via qRT-PCR to determine whether BDNF mRNA levels were enhanced after the different stimulation conditions. Surprisingly, no difference was found in the expression of any of the four BDNF mRNA transcripts (IV activity-dependent, VI activity-dependent, VIII activity-independent and IX total transcripts) examined after any condition (**Figure 1C**). Therefore, neither presynaptic stimulation nor muscle contraction are able to significantly increase *bdnf* mRNA transcription in skeletal muscle under our activity stimulation treatments. These results suggest that the maintenance of pro-BDNF could be due to an increase in BDNF mRNA translation from mRNA pools in the cells.

Because both BDNF and NT4 bind TrkB receptors, we also tested whether synaptic activity with or without muscle contraction regulates NT4 protein level in the skeletal muscle. Results showed that NT4 protein levels did not change under these conditions (presynaptic stimulation: 2.46 ± 1.34, $p > 0.05$; muscle contraction: −7.23 ± 5.84, $p > 0.05$; presynaptic stimulation with contraction: −8.61 ± 5.34, $p > 0.05$), suggesting that activity-dependent enhancement in TrkB-signaling is likely mediated by BDNF.

Muscle Contraction Reduces TrkB.T1 Receptor without Affecting TrkB.FL or p75 Protein Levels

Having established that muscle contraction driven by presynaptic stimulation enhances mBDNF protein levels, we next sought to analyze BDNF receptors that are expressed the most in the skeletal muscle (TrkB.FL, TrkB.T1 and p75). In order to study TrkB receptors, we used an anti-TrkB antibody raised against a peptide sequence corresponding to the amino acids 160–340 of TrkB, the extracellular domain shared by TrkB.FL (145–150 kDa) and TrkB.T1 (95–100 kDa; **Figure 2A**). Furthermore, we confirmed that TrkB.FL and TrkB.T1 bands were only found

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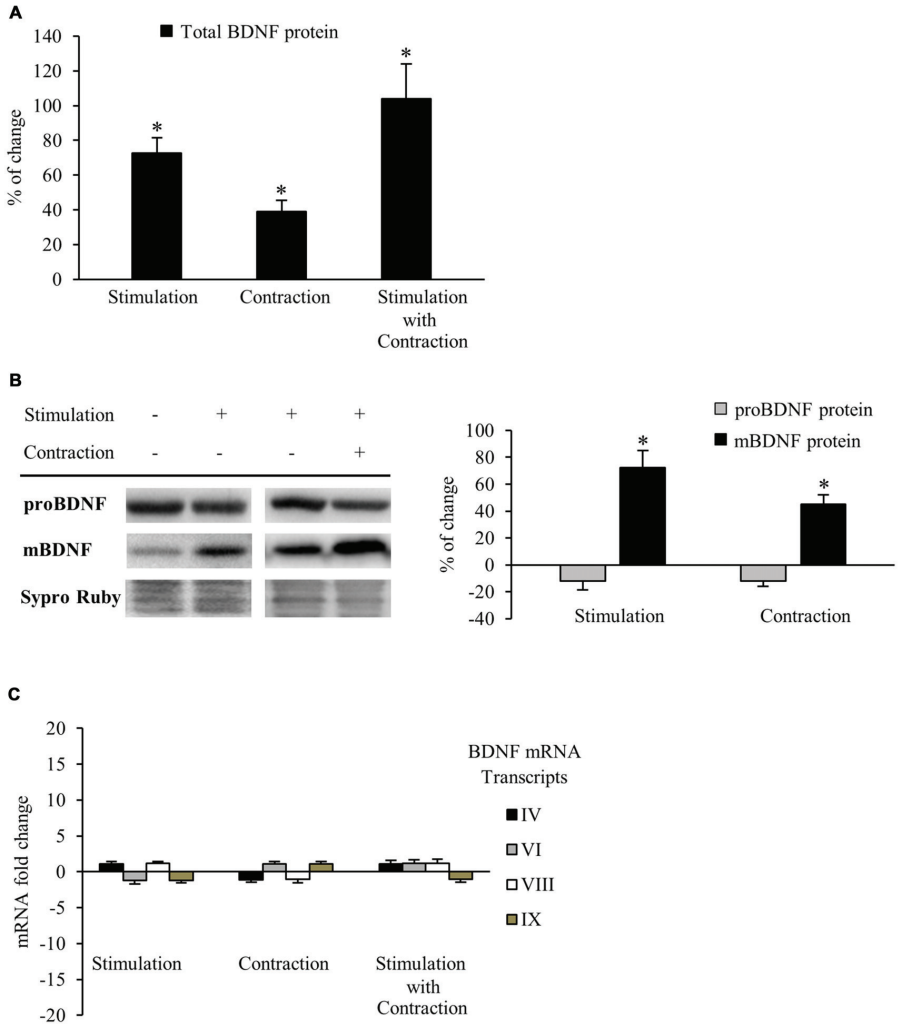

FIGURE 1 | Synaptic activity and muscle contraction increases brain-derived neurotrophic factor (BDNF) protein levels in the diaphragm muscle of rat. (A) ELISA assessment of total BDNF in presynaptic stimulation treatment, contraction treatment and presynaptic stimulation with contraction treatment at 1 Hz stimulation for 30 min. Presynaptic stimulation has been simplified as Stimulation. Each column has been compared to its respective control (see **Table 1**). Data show that both presynaptic stimulation and muscle contraction enhanced BDNF levels. Specifically, presynaptic stimulation significantly increased BDNF protein levels but muscle contraction was able to increase them further. **(B)** Western blot bands and quantification of their optical density show that after stimulation protocols with or without muscle contraction (1 Hz stimulation for 30 min), proBDNF (32 kDa) levels remained the same and mBDNF (14 kDa) increased. **(C)** Quantitative real-time PCR did not show any difference in the expression of *bdnf* mRNA transcripts (IV and VI are activity-dependent, VIII is activity-independent, and IX represents total transcripts) after any stimulation protocol (1 Hz stimulation for 30 min). mRNA data are mean fold change ± SEM, protein data are mean percentage ± SEM, $^*p < 0.05$. Abbreviations: mBDNF, mature BDNF; proBDNF, precursor BDNF.

in the membrane fraction, being absent in the cytosol. In addition, no differences were found between the membrane and total fraction samples (**Figure 2A**). Therefore, to minimize the variability of sample processing, the following results were obtained from total fraction samples. Both receptors were expressed in skeletal muscle at basal conditions, TrkB.T1 being the predominant form (ratio T1/FL = 10.59 ± 0.41, **Figure 2A**). To determine if BDNF receptors are affected by activity, we analyzed how their protein levels were modulated under the stimulation treatments (**Table 1** and **Figure 2B**). We observed

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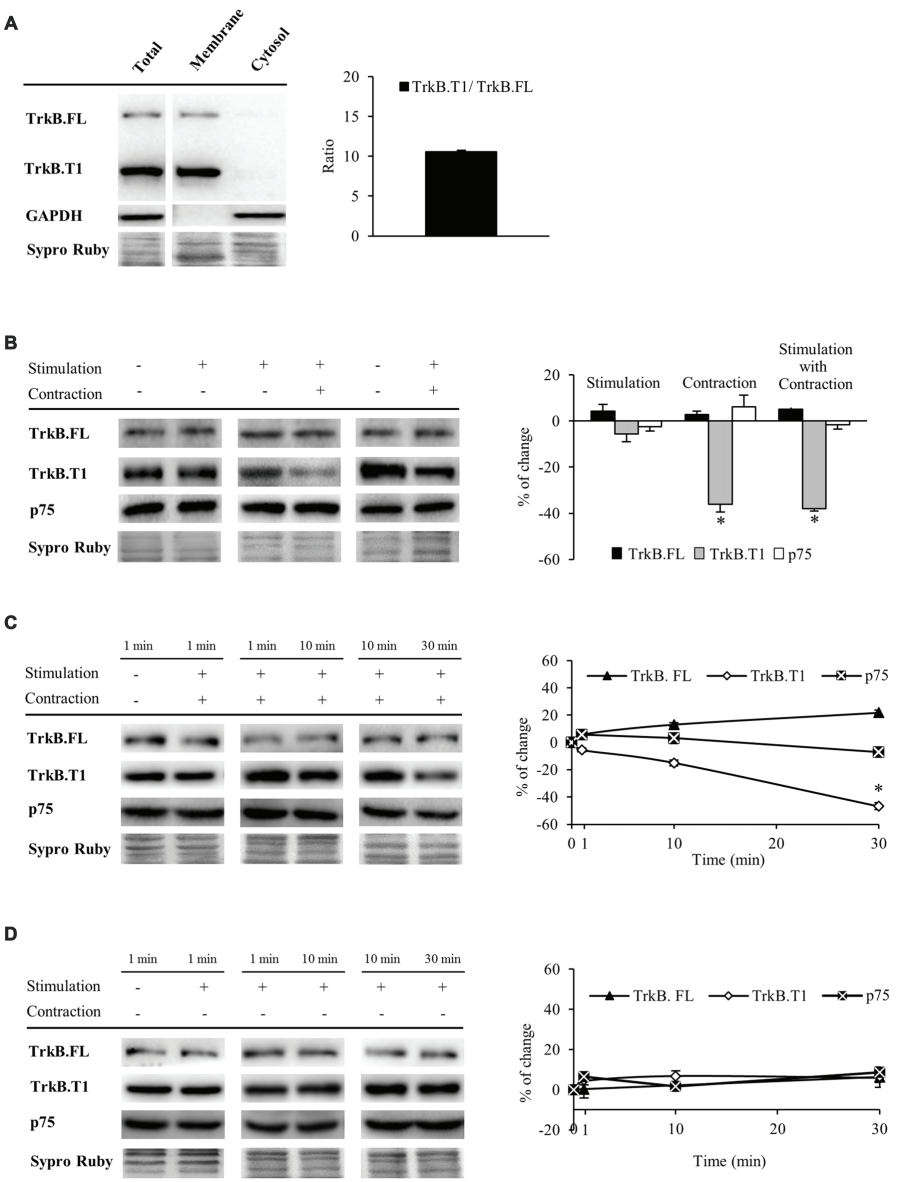

FIGURE 2 | Muscle contraction reduces TrkB.T1 isoform without affecting TrkB.FL or p75 protein levels. Panels **(A–D)** show Western blot bands and their quantification. **(A)** TrkB.FL (145–150 kDa) and TrkB.T1 (95–100 kDa) were present in the total and membrane fractions and, in basal conditions, TrkB.T1 was the predominant form in the skeletal muscle. **(B)** TrkB.FL, TrkB.T1 and p75 receptors in presynaptic stimulation treatment, contraction treatment and presynaptic stimulation with contraction treatment at 1 Hz stimulation for 30 min. Presynaptic stimulation has been simplified as Stimulation. Each column has been compared to its respective control (see **Table 1**). TrkB.T1 was the only receptor modulated by activity, decreasing only when contraction was present. **(C)** TrkB.FL, TrkB.T1 and p75 receptors at 1, 10 and 30 min after presynaptic stimulation with contraction treatment. Each time-point has been compared to its previous time-point. Muscle contraction decreased TrkB.T1 between 10 min and 30 min, without altering TrkB.FL nor p75. **(D)** TrkB.FL, TrkB.T1 and p75 receptors at 1, 10 and 30 min after presynaptic stimulation treatment. Each time-point has been compared to its previous time-point. No receptor was altered without contraction. Data are mean percentage ± SEM, $^*p < 0.05$. Abbreviations: TrkB.FL, Full Length isoform of TrkB; TrkB.T1, truncated isoform 1 of TrkB; p75, low-affinity nerve growth factor receptor.

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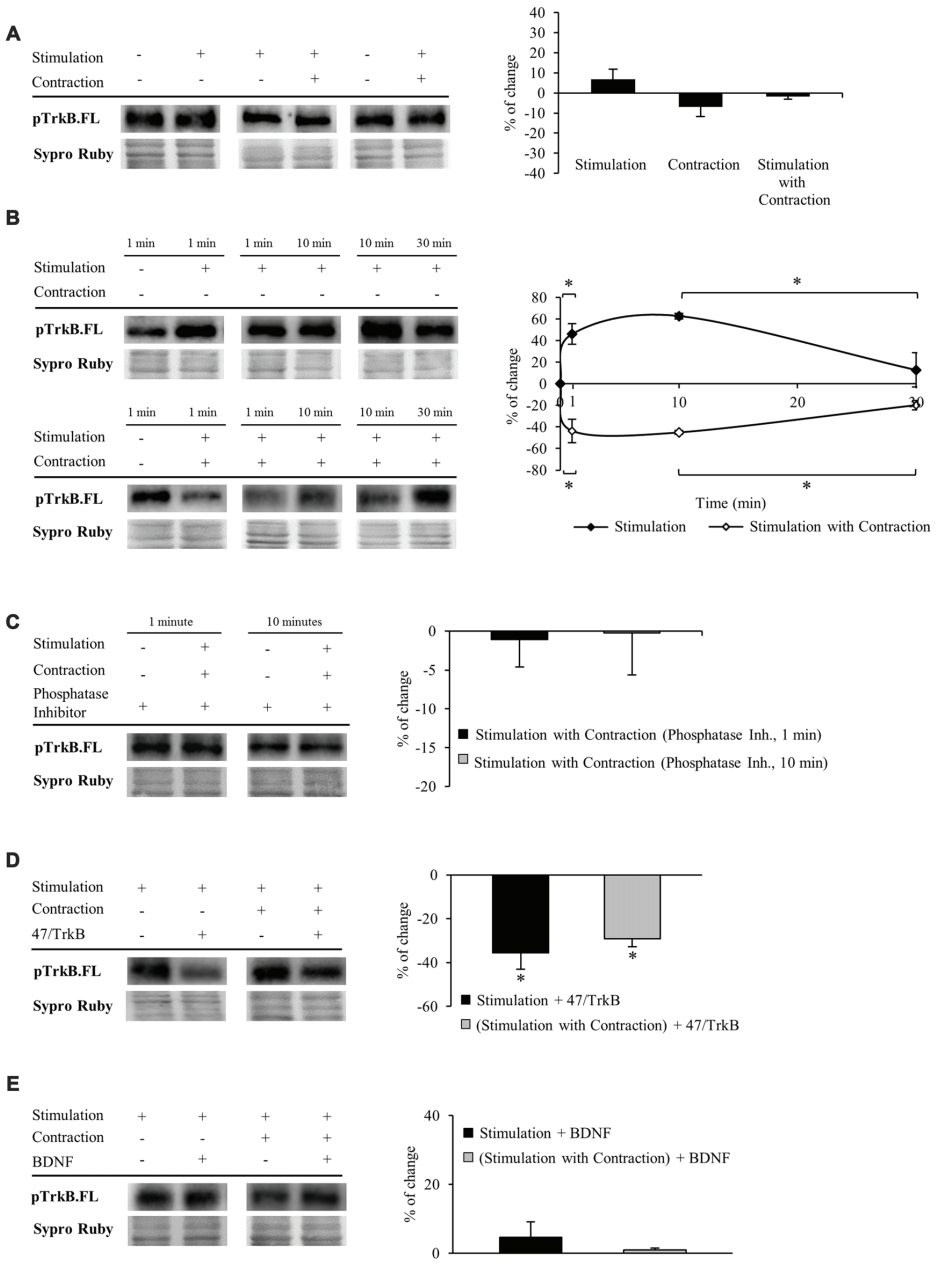

FIGURE 3 | Both synaptic activity and muscle contraction modulates TrkB phosphorylation at tyr816. Panels **(A–E)** show Western blot bands and their quantification. **(A)** pTrkB.FL in presynaptic stimulation treatment, contraction treatment and presynaptic stimulation with contraction treatment at 1 Hz stimulation for 30 min. Presynaptic stimulation has been simplified as Stimulation. Each column has been compared to its respective control (see **Table 1**). pTrkB did not show any change after 30 min of increased activity. **(B)** pTrkB.FL at 1, 10 and 30 min under presynaptic stimulation and presynaptic stimulation with contraction treatments. Each time-point has been compared to its previous time-point. Presynaptic stimulation significantly increased pTrkB.FL at 1 min, an effect maintained after 10 min

(*Continued*)

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FIGURE 3 | Continued
but declined significantly near baseline by 30 min of electrical stimulation. In the presence of muscle contraction, pTrkB.FL levels decreased after 1 min and increased back to baseline levels at 30 min of treatment. **(C)** Phosphatase inhibition prevented the decrease of pTrkB.FL under presynaptic stimulation with contraction at 1 and 10 min. **(D)** Effect of the sequestering antibody 47/TrkB (10 μg/ml) on pTrkB.FL under presynaptic stimulation and presynaptic stimulation with contraction treatments (1 Hz stimulation for 30 min). After both treatments, pTrkB.FL levels decreased. **(E)** Effect of exogenous BDNF (10 μM) on pTrkB.FL following 30 min under presynaptic stimulation and presynaptic stimulation with contraction treatments (1 Hz stimulation for 30 min). pTrkB.FL did not change under these conditions. Specific phosphorylation was determined as the ratio of phosphorylated protein to total protein content. Data are mean percentage ± SEM, $^*p < 0.05$. Abbreviations: pTrkB.FL, phosphorylated TrkB.FL.

that muscle contraction decreased the level of TrkB.T1 protein while presynaptic activity has no apparent effect (−36.13% ± 3.5, $p < 0.05$; −5.58% ± 3.45, $p < 0.05$, respectively). Moreover, none of the stimulation conditions affected TrkB.FL or p75 levels. Thus, the basal FL:T1 ratio was ∼1:11 and contraction treatment elevated it to ∼1:6. Furthermore, preincubation with exogenous BDNF with contraction yielded similar results (TrkB.T1: −24.18% ± 4.3, $p < 0.05$; neither TrkB.FL nor p75 were affected). Therefore, BDNF, which is enhanced by contraction, could contribute to the contraction-dependent decrease of TrkB.T1. However, presynaptic stimulation did not alter the level of any BDNF receptor indicating that muscle contraction *per se* is necessary for synaptic activity to reduce the amount of TrkB.T1 protein.

Since TrkB signaling is known to be quick (Klein et al., 1991; Suen et al., 1997; Takei et al., 1998; Aloyz et al., 1999), before analyzing pTrkB (TrkB phosphorylation), we first analyzed the time course of BDNF receptor levels at 1, 10 and 30 min of stimulation (**Figures 2C,D**). We found that under presynaptic stimulation with contraction, TrkB.FL and p75 protein levels are maintained whereas TrkB.T1 levels start to decrease within 10 min, becoming significant at 30 min of neuromuscular activity (**Figure 2C**). Presynaptic stimulation alone did not alter the levels of any receptor (**Figure 2D**).

Altogether, these results demonstrate that synaptic activity is not enough to modulate TrkB.FL, TrkB.T1 and p75 protein levels even though synaptic activity potently regulates mBDNF levels in the absence of muscle contraction. However, nerve-induced muscle contraction is necessary to downregulate TrkB.T1 levels.

Both Synaptic Activity and Muscle Contraction Modulate TrkB.FL Phosphorylation

We next performed experiments to determine whether TrkB.FL shows enhanced phosphorylation. Specific phosphorylation was determined as the ratio of phosphorylated protein to total protein content (pTrkB.FL/TrkB.FL). We used an antibody which specifically recognizes the tyr816 phosphorylation of TrkB.FL, known to trigger the PLCγ signaling pathway that activates PKC. Unexpectedly, we did not detect any change in TrkB.FL phosphorylation in muscle after 30 min of nerve stimulation, regardless of whether the muscle contracted or not (**Figure 3A**).

This result led us to think that TrkB.FL phosphorylation may be quick and transient. Therefore, we analyzed pTrkB.FL at shorter times of stimulation with and without muscle contraction (1 min and 10 min, 1 Hz). Presynaptic stimulation for 1 min without muscle contraction significantly increased TrkB.FL phosphorylation (46.06% ± 13.36; **Figure 3B**, top), an effect maintained after 10 min of stimulation but declined back to near baseline by 30 min of electrical stimulation. These results suggest that activation of TrkB.FL induced by synaptic activity is very fast and only lasts for a short period of time. Surprisingly, presynaptic stimulation with muscle contraction significantly decreased pTrkB.FL after 1 min (−43.55% ± −15.84; **Figure 3B**, bottom). This reduction in TrkB phosphorylation was also evident at 10 min but returned to baseline levels after 30 min of neuromuscular activity. Interestingly, even shorter periods of presynaptic stimulation with contraction (10 s and 30 s) also significantly decreased pTrkB.FL levels (10 s: −33.49 ± 2.06, $p < 0.05$; 30 s: −41.63 ± 6.49, $p < 0.05$). These results indicate that while synaptic activity without muscle contraction positively regulates TrkB.FL phosphorylation, muscle contraction has the opposite effect, negatively regulating phosphorylation of TrkB.FL on the tyr816 very rapidly, suggesting a quick sequence of activation-phosphorylation-dephosphorylation for TrkB.FL. Because several phosphatases are involved in regulating TrkB signaling (Rusanescu et al., 2005; Ambjørn et al., 2013; Gatto et al., 2013; Ozek et al., 2014), we analyzed whether this decrease of the pTrkB.FL at short times (1 and 10 min) could be prevented by inhibiting phosphatases. Preincubation with a cocktail of phosphatase inhibitors prevented the decrease of the pTrkB.FL (1 min: −1.07 ± 3.52, $p > 0.05$; 10 min: −0.25 ± 5.37, $p > 0.05$; **Figure 3C**). This indicates that phosphatases regulate TrkB.FL phosphorylation of the PLCγ site when muscle contraction occurs following short stimulation periods.

To confirm that phosphorylation of TrkB.FL is ligand-specific, we analyzed the effect of 47/TrkB, an anti-TrkB antibody that extracellularly competes with the binding of endogenous TrkB to any ligand. We found that this inhibitor under presynaptic stimulation, with or without muscle contraction significantly decreases pTrkB.FL after 30 min (−35.61% ± 7.43, $p < 0.05$; −29.24% ± 3.51, $p < 0.05$; respectively, **Figure 3D**), indicating that pTrkB.FL levels are ligand-dependent. Likewise, the increase in TrkB.FL phosphorylation that occurs after 1 min of presynaptic stimulation without muscle contraction was also ligand-dependent, as 47/TrkB reduced significantly pTrkB.FL levels (−32.67% ± 4.84, $p < 0.05$). However, 47/TrkB did not change pTrkB.FL levels after 1 min of presynaptic stimulation with muscle contraction (−1.98% ± 2.92 $p > 0.05$). Possibly, phosphorylation of TrkB.FL in this condition is too low for 47/TrkB to decrease it further.

Similarly, we also determined the level of pTrkB.FL when muscles are incubated in exogenous BDNF at 30 min of stimulation. Unexpectedly, exogenous BDNF did not have any effect on pTrkB level in any condition (presynaptic stimulation

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without muscle contraction: 4.70 ± 4.38 $p > 0.05$; presynaptic stimulation with muscle contraction: 0.99 ± 2.53 $p > 0.05$; **Figure 3E**). 47/TrkB, which blocks endogenous BDNF binding, is able to decrease pTrkB.FL but exogenous BDNF does not increase it. This suggests that TrkB phosphorylation is only induced by endogenous BDNF. However, we cannot discount the possibility that exogenous BDNF could induce an effect through the ratio FL/T1 without the need to induce phosphorylation.

Altogether, these results show that activity modulates BDNF/TrkB signaling in a time-dependent manner. At short times (1–10 min), synaptic activity and muscle contraction regulate phosphorylation of TrkB.FL and, at longer times (30 min), the regulation involves altering the level of TrkB.T1 without effects on TrkB.FL phosphorylation. Within 10 min, presynaptic stimulation induces phosphorylation of TrkB.FL whereas muscle contraction decreases it by the action of phosphatases. These results suggest that muscle contraction performs a regulatory control on the TrkB.FL signaling. After 30 min of stimulation, neither synaptic activity nor muscle contraction has any effect over TrkB.FL phosphorylation. However, this prolonged postsynaptic activity regulates BDNF/TrkB signaling via a reduction of TrkB.T1. Next, we focused on the effects of decreased TrkB.T1 after 30 min of stimulation on the PKCs.

Muscle Contraction Promotes Changes in cPKC Isoforms α and βI through TrkB Receptor

Once we had evaluated how BDNF and its receptors are modulated by activity, we proceeded to examine how this regulation extends to the two presynaptic cPKC isoforms (cPKCα and cPKCβI). TrkB tyr816 phosphorylation directly activates PLCγ1 (Middlemas et al., 1994) which, in turn, activates cPKC through DAG and Ca^{2+}. Moreover, Besalduch et al. (2010) demonstrated that these isoforms are modulated by activity and that muscle contraction has a key role in their upregulation. Our results indicate that presynaptic stimulation resulted in a statistically significant decrease of cPKCα and cPKCβI protein levels (**Figure 4A**, newly reproduced data from Besalduch et al., 2010). This reduction in PKC levels could be due to its activation, and its subsequently degradation (Lee et al., 1996; Lu et al., 1998; Kang et al., 2000; Gould and Newton, 2008; Gould et al., 2009). Thus, we tested whether calcium-dependent PKC is affected by high (5 mM) extracellular Ca^{2+} when combined with presynaptic stimulation. As expected, high Ca^{2+} significantly decreased the level of cPKCα protein (−52.36 ± 2.02, $p < 0.05$) and cPKCβI (−29.15 ± 3.86, $p < 0.05$). Furthermore, we previously demonstrated that MARCKS phosphorylation (PKC's substrate) is increased after presynaptic stimulation (Obis et al., 2015b). These results reinforce the fact that the reduction in cPKCα and cPKCβI promoted by synaptic activity could be due to PKC activation.

Conversely, muscle contraction significantly increased both cPKC isoforms, possibly to provide a pool ready to be activated (**Figure 4A**). These results suggest that a retrograde factor from the muscle cell could influence these isoforms in the nerve terminal. Thus, we proceeded to determine if this modulation is related to BDNF/TrkB signaling. We found that under basal conditions of no stimulation, incubation of exogenous BDNF significantly enhanced cPKCα and cPKCβI protein levels (**Figure 4A**), mimicking the effect of muscle contraction.

Exogenous BDNF decreased cPKCα and cPKCβI protein levels (−41.96% ± 3.66 $p < 0.05$; −34.21% ± 7.77 $p < 0.05$; respectively) under presynaptic stimulation (**Figure 4B**). This result may indicate that TrkB signaling reduces total cPKC levels in this condition, possibly due to an increase in their activation and subsequent degradation. In concordance, 47/TrkB preincubation reversed the effects of presynaptic stimulation, increasing cPKCα and cPKCβI protein levels a 49.10% ± 6.90 and a 69.10% ± 15.6, respectively ($p < 0.05$; **Figure 4B**). This increase could be explained because 47/TrkB inhibits TrkB signaling and decreases cPKC activation and their subsequent degradation.

On the other hand, under presynaptic stimulation with muscle contraction, exogenous BDNF further enhanced the increase of cPKCα and cPKCβI protein levels (30.83% ± 17.60, $p < 0.05$; 21.78% ± 11.09, $p < 0.05$, respectively; **Figure 4C**). In concordance, incubation with 47/TrkB completely reversed the effects produced by presynaptic stimulation with contraction, decreasing cPKCα and cPKCβI protein levels (−44.40% ± 10.0, $p < 0.05$; −37.60% ± 11.10, $p < 0.05$, respectively; **Figure 4C**). Thus, these results indicate that muscle contraction can enhance the levels of cPKCα and cPKCβI through BDNF/TrkB signaling, reverting the synaptic-induced downregulation of cPKC isoforms. Interestingly, at this point, muscle contraction significantly decreases TrkB.T1 levels without changing TrkB.FL. Therefore, the balance between TrkB.FL/TrkB.T1 could enhance cPKC synthesis or, alternatively, could inhibit its activity-induced degradation, increasing the total levels to revert the effect of synaptic activity.

Altogether, these results demonstrate a direct link between activity, BDNF/TrkB signaling and cPKCα and cPKCβI protein levels. In brief, BDNF regulation is directed by both synaptic activity and muscle contraction in opposite directions, emphasizing a key role of nerve-induced muscle contraction in the modulation of presynaptic cPKCα and cPKCβI isoforms through TrkB.

Phosphorylation of cPKC Isoforms (cPKCα and cPKCβI) is Regulated by Neuromuscular Activity and TrkB

Activation of PKC isoforms requires phosphorylation (Newton, 2003). Therefore, our next objective was to determine whether neuromuscular activity affects phosphorylation of cPKCα and cPKCβI isoforms. Measurements of a protein's phosphorylation status may change through modification of the individual proteins phosphorylation level (Tremblay et al., 2007) and/or by alterations within the total amount of protein available (Yung et al., 2011). As such, we show specific phosphorylation determined as the ratio of phosphorylated protein to total amount of protein available, but also as the individual proteins phosphorylation level (**Figure 5**).

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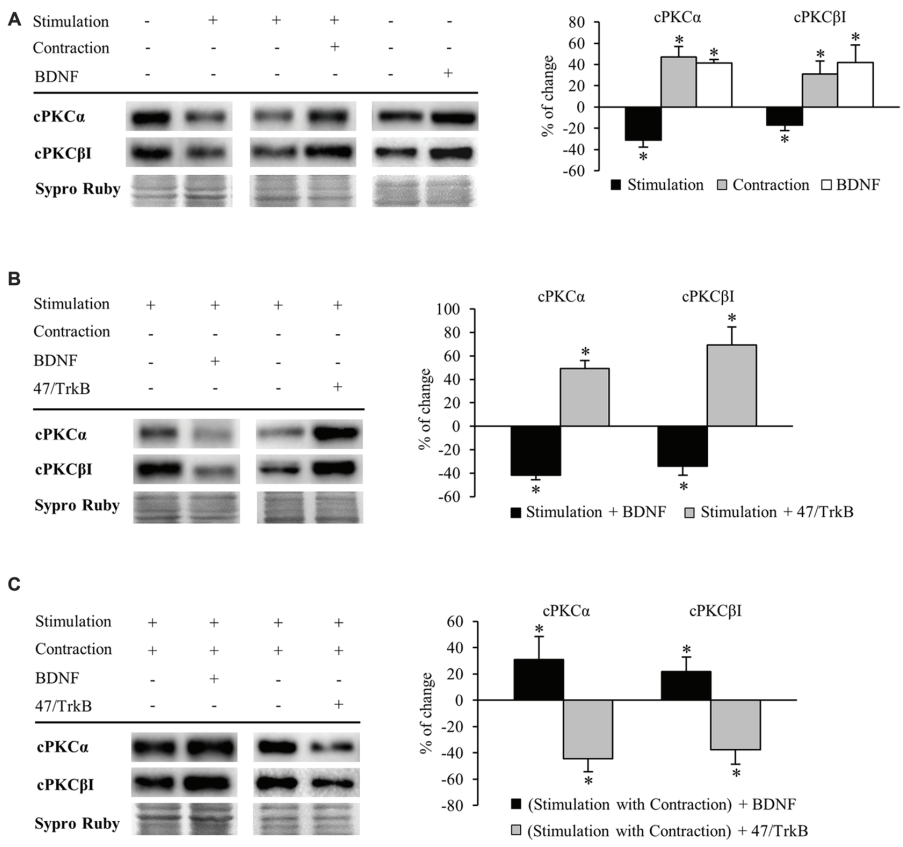

FIGURE 4 | Synaptic activity and muscle contraction modulates cPKC isoforms through BDNF/TrkB signaling. Panels **(A–C)** show Western blot bands and their quantification. **(A)** cPKC isoforms α and βI in presynaptic stimulation treatment and contraction treatment at 1 Hz stimulation for 30 min. cPKC α and βI in basal conditions with exogenous BDNF for 30 min. Presynaptic stimulation has been simplified as Stimulation. Each column has been compared to its respective control (see **Table 1**). Results show that presynaptic stimulation decreased the levels of cPKC α and βI whereas muscle contraction increased them. In basal conditions, exogenous BDNF increased the levels of cPKC α and βI. **(B)** Effect of exogenous BDNF (10 μM) and 47/TrkB (10 μg/ml) on cPKC isoforms α and βI in presynaptic stimulation treatment. BDNF enhanced the effects of presynaptic stimulation, decreasing cPKC levels, and conversely, 47/TrkB increased them. **(C)** Effects of exogenous BDNF (10 μM) and 47/TrkB (10 μg/ml) on cPKCα and cPKCβI in presynaptic stimulation with contraction treatment. BDNF enhanced the effects of presynaptic stimulation with contraction treatment, increasing cPKC levels, and conversely, 47/TrkB decreased them. Data are mean percentage ± SEM, $^*p < 0.05$. Abbreviations: cPKCα, conventional Protein Kinase C (PKC) α; cPKCβI, conventional PKC βI.

Presynaptic stimulation without muscle contraction resulted in a statistically significant increase of the ratio of phosphorylated forms of cPKCα (98.47% ± 4.33, $p < 0.05$) and cPKCβI (72.26% ± 2.07, $p < 0.05$; **Figure 5A**). This increase in pPKC levels indicates that presynaptic stimulation enhances directly phosphorylation of cPKC. Moreover, this increase in pPKC levels reaffirms the fact that the total PKC decrease described above is caused by an activation-induced degradation. Furthermore, muscle contraction does not change the ratio of pPKC/PKC either cPKCα or cPKCβI (**Figure 5A**). However, the individual phosphorylated cPKCα and cPKCβI levels are increased after muscle contraction (pPKCα 36.95% ± 8.13, $p < 0.05$; pPKCβI 42.98% ± 6.17, $p < 0.05$; **Figure 5A**) similarly than cPKCα and cPKCβI (**Figure 4**). In this condition, where total cPKC are increased, muscle contraction would inhibit activity-dependent degradation or, alternatively, enhance cPKC synthesis, establishing a larger pool of cPKCs isoforms ready to be phosphorylated and activated. This may indicate that muscle contraction enhances phosphorylation of cPKCs by means of increase of total protein PKC protein level. Moreover, we found that under basal conditions of no stimulation with exogenous BDNF, pPKCα and pPKCβI protein levels remain the same (**Figure 5A**).

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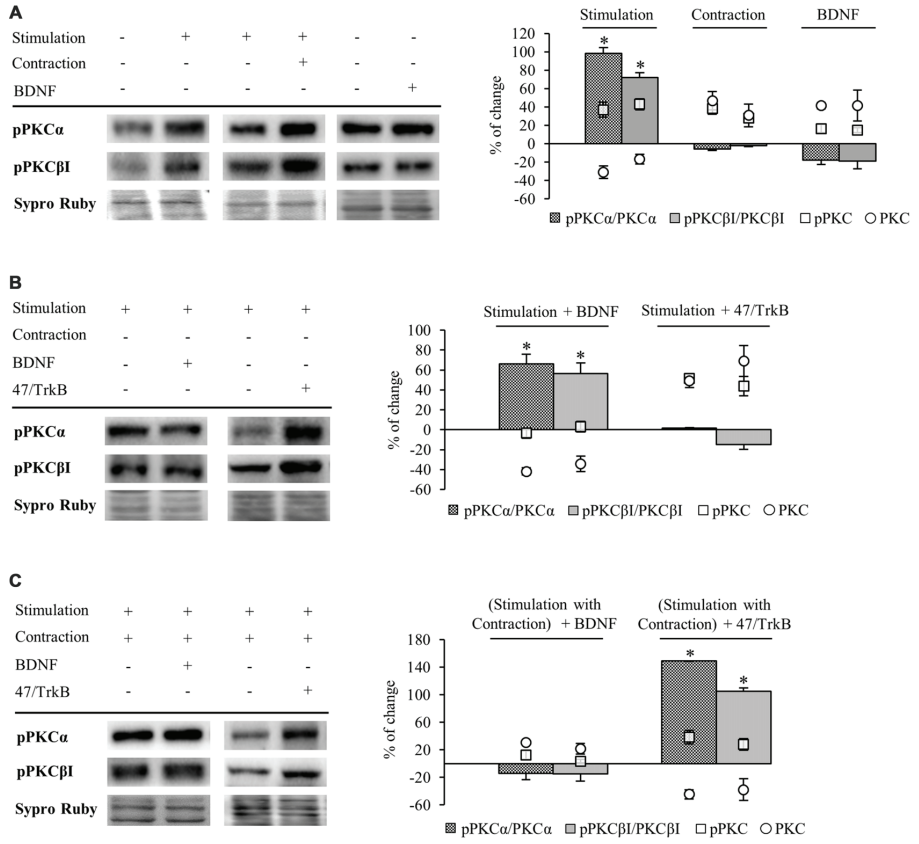

FIGURE 5 | Phosphorylation of cPKC isoforms is regulated by activity and TrkB. Panels **(A–C)** show Western blot bands and their quantification. **(A)** pPKCα and pPKCβI in presynaptic stimulation treatment and contraction treatment at 1 Hz stimulation for 30 min. pPKCα and pPKCβI in basal conditions with exogenous BDNF for 30 min. Presynaptic stimulation has been simplified as Stimulation. Each column has been compared to its respective control (see **Table 1**). Results show that presynaptic stimulation increases the ratio of phosphorylated forms of cPKCα and cPKCβI. However, muscle contraction does not change the ratio of pPKC/PKC but increases the individual phosphorylated cPKCα and cPKCβI. Furthermore, the ratio of phosphorylated forms of cPKCα and cPKCβI did not show any change under basal conditions of no stimulation with exogenous BDNF. **(B)** Effect of exogenous BDNF (10 μM) and 47/TrkB (10 μg/ml) on pPKC isoforms α and βI in presynaptic stimulation treatment. Incubation with exogenous BDNF led to a significant increase of the ratio of pPKCα and pPKCβI (due to a decrease in the total amount of total cPKCα and cPKCβI). Incubation with 47/TrkB does not affect the ratio because the increase in the individual phosphorylated PKC is a consequence of the increase in total PKC. **(C)** Effects of exogenous BDNF (10 μM) and 47/TrkB (10 μg/ml) on pPKCα and pPKCβI in presynaptic stimulation with contraction treatment. Incubation with exogenous BDNF slightly decreases pPKC/PKC for either cPKCα and cPKCβI (with an accompanied increase in the total amount of PKC and the maintenance of pPKCα and pPKCβI). Incubation with 47/TrkB significantly increases the ratio of cPKCα and cPKCβI phosphorylation. In concrete, the decrease in total PKC is accompanied by an increase in the individual phosphorylation of PKC. Specific phosphorylation was determined as the ratio of phosphorylated protein to total protein content, but also as the individual protein phosphorylation level. Data are mean percentage ± SEM, $^*p < 0.05$. Abbreviations: cPKCα, conventional- PKC α; cPKCβI, conventional- PKC βI; pPKCα, phosphorylated PKC α; pPKCβI, phosphorylated PKC βI.

We next determined the involvement of BDNF/TrkB pathway on pPKC levels. Preincubation with exogenous BDNF under presynaptic stimulation increases the ratio of pPKCα (66.32% ± 9.53, $p < 0.05$) and pPKCβI protein levels (56.50% ± 10.47, $p < 0.05$; **Figure 5B**). However, the increase in the ratio is due to a decrease in the total amount of total cPKCα and cPKCβI, indicating that the phosphorylated pool is maintained and exogenous BDNF does not promote PKC phosphorylation. Moreover, 47/TrkB does not affect the ratio because the increase in the individual phosphorylated PKC is a consequence of the increase in total PKC (**Figure 5B**). On the other hand, under presynaptic stimulation with muscle contraction, exogenous BDNF slightly decreases pPKC/PKC either cPKCα and cPKCβI. These decreases are due to an increase in the total amount of PKC with an accompanied maintenance

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of pPKCα and pPKCβI (**Figure 5C**). Thus, confirming that the contraction thought BDNF increase PKC synthesis rather than PKCs phosphorylation. However, incubation with 47/TrkB significantly increases the ratio of cPKCα and cPKCβI phosphorylation (149.01% ± 10.11, $p < 0.05$; 105.22% ± 8.86, $p < 0.05$, respectively; **Figure 5C**). In concrete, the decrease in total PKC is accompanied by an increase in the individual phosphorylation of PKC (38.32% ± 6.05, $p < 0.05$) and PKCβI (28 ± 4.92, $p < 0.05$). This might indicate that the activity-dependent ratio FL/T1 could decrease pPKCs due to an activity-dependent pPKC translocation, action and subsequent degradation.

So, an increase of the synaptic activity results in an increase of the phosphorylation of cPKCα and cPKCβI isoforms, whose activity would be enhanced by TrkB/PLCγ signaling pathway, decreasing total cPKCα and cPKCβI levels due to their subsequent degradation. Muscle contraction, through an increase in TrkB FL/T1 ratio would inhibit PKC degradation or enhance their synthesis, increasing the total PKC levels ready to be phosphorylated to revert the downregulating effect of the synaptic activity.

cPKCβI Is Involved in ACh Release in the NMJ

PKC is an important family of kinases that regulates neurotransmission at the NMJ (Hori et al., 1999; Santafé et al., 2005, 2006). In particular, PKC is activated and regulates the release of ACh when electrical stimulation is applied to the nerve (Besalduch et al., 2010). However, it is unknown which isoform of PKC is involved in this regulation. cPKCα and cPKCβI are differently distributed in the NMJ with cPKCα located in the nerve terminal, muscle cell and terminal Schwann cells but cPKCβI is exclusively located in the nerve terminal (Besalduch et al., 2010). When we examine the expression of cPKCβI based on immunofluorescent labeling of semithin cross-sections of LAL muscles (**Figure 6A**), we find cPKCβI fine granular green immunofluorescence located over the postsynaptic line of the nicotinic acetylcholine receptor (nAChR) site (in red) and colocalized with the neurofilament and syntaxin (NF+Synt, in blue top image 1) in the nerve terminal. cPKCβI is not colocalized with labels for either Schwann cells (S100, in blue bottom image 2) or AChRs postsynaptically (in red). These results confirm the exclusive presynaptic localization of cPKCβI and makes it a good candidate to regulate neurotransmitter release.

To test this idea directly, we used an isozyme-selective translocation peptide inhibitor (βIV5–3; Liu et al., 1999; Zhang et al., 2015) to inhibit cPKCβI activity. Western blotting confirmed that βIV5–3 decreases the ratio of phosphorylated cPKCβI levels (−36.95% ± 7.60, $p < 0.05$; **Figure 6B**) in the membrane fraction of rat diaphragm muscles. Electrophysiological experiments in presynaptic stimulated muscles (contraction blocked) incubated with βIV5–3 in the dose range commonly used (1, 5, and 10 μM, 1 h incubation) revealed a significant reduction of EPP amplitude, indicating significantly less ACh release (**Figure 6C**). Altogether, these results highlight the key role of cPKCβI in neurotransmission in NMJ.

DISCUSSION

In the neuromuscular system, evidence supports BDNF/TrkB signaling as a regulator of neuromuscular function. However, it remained unknown if nerve-induced muscle contraction *per se* can modulate crucial aspects of neuromuscular synaptic function through BDNF and its receptor, TrkB. The results of the present study demonstrate that nerve induced-muscle activity is a key regulator of BDNF/TrkB signaling pathway, activating presynaptic cPKC isoforms (in particular cPKCβI) to modulate synaptic function.

Diaphragm muscle has been described as a useful model of flat skeletal muscle to study synaptic function (Rosato Siri and Uchitel, 1999; Urbano et al., 2003; Besalduch et al., 2010; Chand et al., 2015; Obis et al., 2015b). Accessibility to the phrenic nerve helps to dissect and stimulate it and thus enhance independently synaptic activity and muscle contraction. Diaphragm is in a way unusual because only a subset of muscle fibers is active with a prolonged duty cycle as compared to other skeletal muscles (Mantilla et al., 2004, 2014; Seven et al., 2014). However, nerve stimulation simultaneously recruits all motor units and uniforms the heterogeneous level of activity of the fibers.

Nerve-Induced Muscle Contraction Enhances the Activity-Dependent Increase of mBDNF and Downregulates TrkB.T1 Levels in Skeletal Muscle

Exercise training increases BDNF expression in spinal cord and in skeletal muscle in rodents (Gómez-Pinilla et al., 2001, 2002; Cuppini et al., 2007; Zoladz and Pilc, 2010; Gomez-Pinilla et al., 2012) and basal levels of neuromuscular activity are required to maintain normal levels of BDNF in the neuromuscular system (Gómez-Pinilla et al., 2002). However, previous studies did not explore if BDNF expression in muscle was enhanced by synaptic activity independent of muscle contraction or whether muscle contraction was also necessary. Here we show that both activities can increase muscle BDNF levels, with muscle contraction being able to increase levels over and above what nerve transmission alone can enhance. Several findings suggest the muscle cell as a source of BDNF. Although BDNF is found in the three cells at the NMJ (Garcia et al., 2010), *bdnf* mRNA is only located inside myocytes (Liem et al., 2001), and not in the presynaptic region of the axon. Moreover, BDNF is produced by contracting myotubes *in vitro* (Matthews et al., 2009). By using μ-CgTx-GIIIB we are able to block myofibril contraction without altering ACh signaling. We show here that the increase of BDNF levels induced by presynaptic stimulation could arise from the presynaptic region (due to the electrical stimulus) and/or in the postsynaptic region (due to local ACh signaling). However, the greater increase of BDNF levels when both nerve and muscle are active compared to

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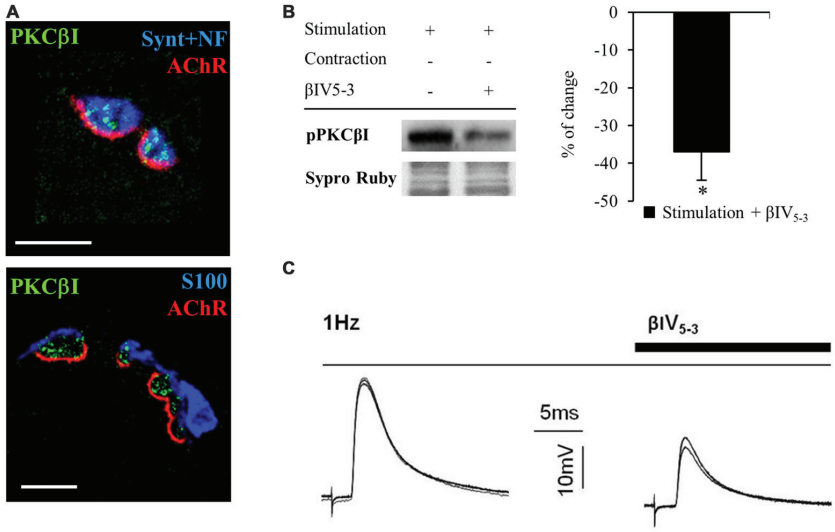

FIGURE 6 | cPKCBI is involved in ACh release in the neuromuscular junction (NMJ). (A) Semithin cross-sections from whole-mount multiple-immunofluorescent stained levator auris longus (LAL) muscles. (**A**, top) Triple staining labeled cPKCβI (in green), AChRs (fluorescent α-BTX in red) and nerve terminal (with anti-syntaxin and anti-neurofilament antibodies in blue). (**A**, bottom) Triple staining labeled cPKCβI in green, AChRs in red and Schwann cell (with an anti S100 antibody in blue). The images show the cPKCβI immunolabel located only between Schwann cell and muscle cell and well colocalized with the nerve terminal, indicating that cPKCβI is exclusively located at the presynaptic component. The scale bars indicate 2.5 μm. **(B)** Western blot bands and their quantification show the effect of the selective translocation peptide inhibitor (βIV5–3, 10 μM) on pPKCβI in the membrane fraction in presynaptic stimulation treatment. Incubation with βIV5–3 decreased the phosphorylated cPKCβI in the membrane fraction, thus confirming its inhibitory effect. Data are mean percentage ± SEM, $^*p < 0.05$. **(C)** Intracellular recordings show evoked Endplate potentials (EPPs) in basal conditions (1 Hz) and after incubation with βIV5–3. Raw data indicate that ACh release is reduced by incubation with βIV5–3, highlighting the involvement of cPKCβI in neurotransmitter release. Abbreviations: LAL, levator auris longus; α-BTX, α-bungarotoxin; cPKCβI, conventional-PKC βI; pPKCβI, phosphorylated PKC βI; EPP, end-plate potential.

when only the synapse is active strongly suggests a postsynaptic origin through *bdnf* mRNA translation that is directly linked to myofibril contraction (represented as via 2 in **Figure 7**). Moreover, evidence indicates that neurotrophins are released acutely following neuronal depolarization (Griesbeck et al., 1999; Mowla et al., 1999; Goggi et al., 2003). In fact, direct activity-dependent pre- to post-synaptic transneuronal transfer of BDNF has recently been demonstrated using fluorescently-labeled BDNF (Kohara et al., 2001). Moreover, electrical stimulation can activate Schwann cells (SCs) to secrete BDNF, which requires the involvement of calcium influx (Luo et al., 2014). Because the well-known interactions between Schwann cell and the nerve terminal (Todd et al., 2007), some involvement of the glial cell in the responses we observed would be not discarded. Thus, although the source and the realize of BDNF is not clear, our results show that activity in either the synapse or the muscle increase mature BDNF in skeletal muscle.

Previous results indicate that *bdnf* mRNA increases in skeletal muscle after several days of increased physical activity (Gómez-Pinilla et al., 2001, 2002; Cuppini et al., 2007; Zoladz and Pilc, 2010; Gomez-Pinilla et al., 2012). However, we find that 30 min of synaptic activity with or without contraction was not sufficient to increase *bdnf* mRNA in muscle, suggesting that short-term acute neuromuscular activity enhances muscle BDNF levels by promoting its translation and/or maturation (e.g., through increased protease activity), allowing a build-up of mBDNF and leaving net pro-BDNF levels unchanged.

BDNF isoforms, pro-BDNF and mBDNF, bind distinct receptors to mediate divergent neuronal actions (Lu, 2003; Woo et al., 2005; Hempstead, 2006; Yang et al., 2009; Je et al., 2013). Pro-BDNF interacts preferentially with p75, whereas mBDNF selectively binds and activates its specific receptor TrkB. There are several alternatively spliced isoforms of TrkB with the same affinity to neurotrophins, including TrkB.FL and two truncated TrkB isoforms T1 and T2 (TrkB.T1 and TrkB.T2), which lack part of the intracellular kinase domain (Middlemas et al., 1991; Reichardt, 2006). Evidence suggests that heterodimers of TrkB.FL with the truncated isoforms inhibit trans-autophosphorylation of TrkB.FL, reducing BDNF signaling (Eide et al., 1996; Baxter et al., 1997; Rose et al., 2003; Dorsey et al., 2012; Wong and Garner, 2012). TrkB.T2 is a variant mainly predominant in the brain tissue and does not appear to have individual signaling ability (Stoilov et al., 2002). TrkB.T1 is the main truncated isoform in the skeletal muscle and some studies suggest unique signaling roles for TrkB.T1, for example, by modulating Ca^{2+} signaling mechanisms (Rose et al., 2003).

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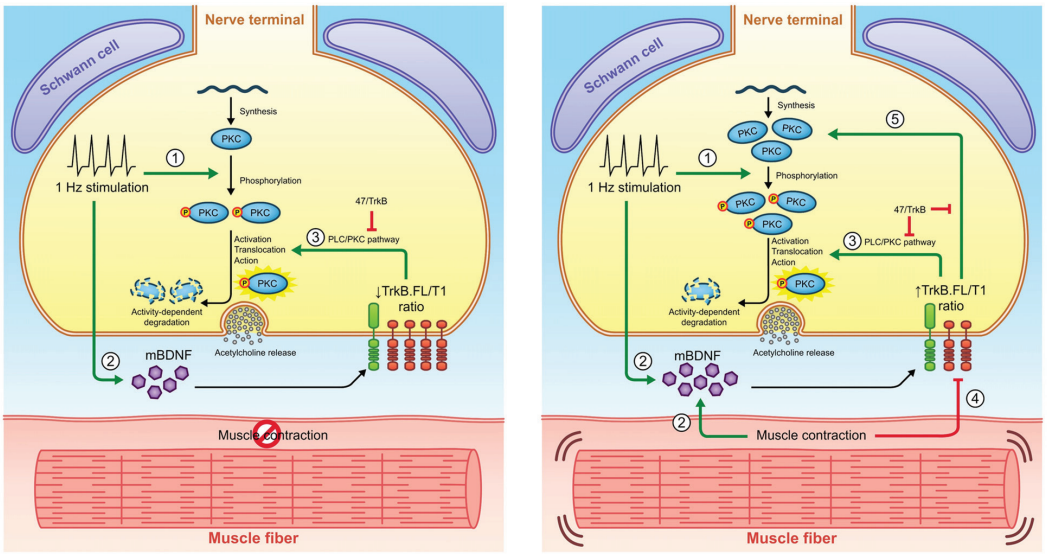

FIGURE 7 | Retrograde communication from myocytes to axon terminals through BDNF/TrkB signaling. (A) Graphical representation of BDNF signaling outcomes observed under presynaptic stimulation without muscle contraction and presynaptic stimulation with muscle contraction. Presynaptic activity (1) promotes cPKC phosphorylation and (2) increases BDNF levels. BDNF acts presynaptically through (3) TrkB/PLCγ/PKC pathway to complete the activation of pPKC and thus enhance synaptic vesicle fusion and ACh release. Once PKC have executed their action, they are typically degraded thus decreasing its protein levels. **(B)** Graphical representation of BDNF signaling outcomes observed under presynaptic stimulation with contraction. The presence of contraction (2) further increases BDNF protein levels and (4) decreases TrkB.T1 protein levels, ultimately increasing the ratio TrkB.FL/T1. Even though this ratio still enhances (3) cPKC activity and its subsequent degradation, it promotes the increase in total PKC protein levels (5). Presumably, this might be due to an increase in PKC synthesis or alternatively, to a decrease in their activity-induced degradation. Consequently, pPKC levels are enhanced. The influence of TrkB.FL/T1 ratio over cPKC in both conditions is demonstrated by the sequestering antibody 47/TrkB. Therefore, muscle contraction could modulate BDNF/TrkB signaling to promote a retrograde regulatory feedback from the myocyte that maintains the levels of presynaptic cPKC. This may have an impact in NMJ functionality since cPKCβI activity is required for acetycholine release.

Other evidence suggests that TrkB.T1 acts in a dominant negative fashion to decrease signaling through TrkB.FL (Eide et al., 1996; Gonzalez et al., 1999; Haapasalo et al., 2001; Dorsey et al., 2012). In concordance with previous findings (Dorsey et al., 2012), we show that TrkB.T1 levels predominate over TrkB.FL isoform 11:1 in the resting NMJ (**Figure 2A**). We also found that muscle contraction downregulates TrkB.T1 (decreasing the ratio to 6:1) without changing TrkB.FL or p75 levels (represented as via 4 in **Figure 7**). Therefore, it appears that the ratio between TrkB.FL and TrkB.T1 could determine the net effect of BDNF signaling at neuromuscular system.

BDNF binding to the TrkB.FL activates the intrinsic tyrosine kinase domain, leading to autophosphorylation in the activation loop (tyr701, tyr706 and tyr707; Guiton et al., 1994; Reichardt, 2006). The phosphorylation of these residues can leads to the transphosphorylation of others tyrosine residues (Cunningham et al., 1997; Friedman and Greene, 1999) being tyr515 and tyr816 the most extensively studied phosphorylation sites (Middlemas et al., 1994; Segal et al., 1996). pTyr515 interacts with Shc or Frs2 and provides a mechanism for the activation of the: (i) Ras–mitogen-activated protein kinase (Ras/MAPK) pathway; and (ii) phosphatidylinositol-3 kinase—Akt pathway (PI-3K/Akt). On the other hand, pTyr816 links TrkB receptors to the (iii) phospholipase Cγ gamma (PLC-γ) pathway (Obermeier et al., 1993). Although the other signal transduction pathways activated by TrkB could be involved in BDNF response (e.g., due to phosphorylation of tyr515), activation of PLCγ is one attractive candidate to mediate synaptic potentiation by PKC because its activation would result in intracellular Ca^{2+} release via the second messenger IP_3 (Obermeier et al., 1993). Moreover, it is known that, in most cells, M_1, A_1, (autoreceptors of the principal transmitter and cotransmitter product adenosine) and TrkB receptors among others, cooperate by stimulating PLCγ pathway. In this regard, it should also take into account the possible involvement of M_1, A_1 in PKC activation in the NMJ.

Neuronal activity has been shown to rapidly activate TrkB and potentiate its signaling, an effect attributed to activity-dependent secretion of BDNF (Meyer-Franke et al., 1998; Aloyz et al., 1999; Patterson et al., 2001). However, Du et al. (2003) reported that the activity-dependent enhancement of

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TrkB tyrosine kinase in cultured hippocampal neurons is not due to elevated BDNF secretion. Moreover, it has been demonstrated that the responsiveness of TrkB phophorylation to BDNF is reduced after the 2nd week postnatally in rat brain microslices at sites tyr816, tyr515 and tyr705/6 (Di Lieto et al., 2012). In our experiments, exogenous BDNF did not increase the tyr816 phosphorylation of TrkB.FL, known to trigger the PLCγ signaling pathway that activates PKC. Nevertheless, we found that presynaptic stimulation induces a quick increase in pTrkB.FL after 1–10 min but returned to baseline by 30 min (**Figure 3B**). On the one hand, the rapid phosphorylation of TrkB (1 min) that we found, suggests that the action of BDNF is particularly fast. This could be due to a quick extracellular diffusion of BDNF or to a site of release close to the NMJ. Both possibilities could explain the short time course of presynaptic TrkB activation. On the other hand, the subsequent decrease in the phosphorylation of TrkB could be caused by a decrease in the phosphorylation process or by phosphatase activity. We found support for enhanced phosphatase activity: muscle contraction at short times decreases phosphorylation of TrkB.FL by increasing phosphatase activity (**Figures 3B,C**). Previous work revealed the complexity of phosphatase control and showed that endogenous protein-tyrosine phosphatases negatively control BDNF sensitivity, antagonizing tyrosine phosphorylation of TrkB (Rusanescu et al., 2005; Ambjørn et al., 2013; Gatto et al., 2013; Ozek et al., 2014). The mechanism by which muscle contraction decreases TrkB phosphorylation (tyr 816) at short times at the NMJ needs further investigation. This mechanism could exert a retrograde regulatory control over the BDNF/TrkB.FL signaling to modulate presynaptic BDNF/TrkB action. In conclusion, the modulation of BDNF/TrkB signaling by neuromuscular activity is time-dependent at the NMJ. At short times (1–10 min), synaptic activity and muscle contraction regulate phosphorylation of TrkB.FL (tyr816) and, at longer times (30 min), the regulation is mediated by an effect on TrkB.T1.

Muscle Contraction Induced by Nerve Electrical Activity Promotes Changes in cPKC Isoforms through BDNF/TrkB Pathway

Several studies demonstrated that BDNF-induced potentiation of presynaptic vesicle release requires TrkB phosphorylation and PLC activation (Kleiman et al., 2000), which activates PKC. When PKCs are activated (phosphorylated and anchored to the membrane) they enhance vesicle fusion and ACh release (West et al., 1991; Numann et al., 1994; Byrne and Kandel, 1996; Besalduch et al., 2010; Lanuza et al., 2014).

Recently, we reported that synaptic activity induces changes in the presynaptic expression of the novel PKCε through TrkB receptor at the NMJ (Obis et al., 2015a). As previously published (Besalduch et al., 2010), here we also show that cPKCα and cPKCβI are downregulated by presynaptic stimulation (represented as via 3 in **Figure 7**). In this presynaptic stimulation condition, MARCKS phosphorylation levels (PKC's substrate) are increased, an indicator of PKC activation (Obis et al., 2015b). Once PKCs are activated and have executed their action, PKCs are down-regulated in an activation-dependent manner, a process mediated by the proteasome (Lee et al., 1996; Lu et al., 1998; Kang et al., 2000; Gould and Newton, 2008; Gould et al., 2009). As we show here, high Ca^{2+} conditions induce a decrease in calcium dependent cPKCα and cPKCβI protein levels, indicating that they are activated and then degraded without being replaced by newly synthesized cPKCs.

In contrast, muscle contraction increases cPKCα and cPKCβI protein levels through TrkB and reverses the downregulation induced by the synaptic activity. This could be associated with an increased protein synthesis (represented as via 5 in **Figure 7**) or decreased protein degradation (represented as via 3 in **Figure 7**). As far as we know, the mechanism of the synthesis of cPKC has not been extensively studied. However, it is known that BDNF has effects on the proteome and it may be due to changes in transcription activity (e.g., activating CREB; Finkbeiner et al., 1997; Groth and Mermelstein, 2003; Caldeira et al., 2007). Moreover, it may be due to a direct regulation of the translation machinery through the mammalian target of rapamycin (mTOR) pathway (Takei et al., 2001). A plausible hypothesis could be that some of these signaling pathways could be also involved in the BDNF induced-enhancement of presynaptic cPKC at the NMJ.

cPKCα and cPKCβI are presynaptically located, with cPKCβI being exclusively located at the nerve terminal (Besalduch et al., 2010). Hence, the modulation of presynaptic cPKCα and cPKCβI may require a neurotrophic positive feedback generated by the postsynaptic contractile activity, BDNF being a possible mediator. It has been suggested that neuromuscular activity increases retrograde transport of BDNF from the muscle to the spinal cord (Yan et al., 1992; Koliatsos et al., 1993; Curtis et al., 1998; Sagot et al., 1998).

Moreover, our results demonstrate that synaptic activity and muscle activity have opposite effects on cPKC protein level and these effects are mediated by the increased endogenous BDNF (induced by pre- and postsynaptic activities) through TrkB. This suggests distinct roles of presynaptic vs. postsynaptic induced-BDNF. Moreover, exogenous BDNF enhances the opposite effects of presynaptic and postsynaptic activities on cPKC protein levels through a different ratio of TrkB.FL/T1. Muscle contraction increases TrkB.FL/T1 ratio and the reduction of the T1 dominant negative fashion over FL signaling upregulates presynaptic cPKCs. Therefore, the apparent distinct roles of the pre- and postsynaptic BDNF are consequence of the muscle contraction-induced decrease of TrkB.T1. This TrkB.T1 downregulation could be related with the recycling events of the receptor. TrkB isoforms seem to be differentially recycled after BDNF-induced endocytosis with TrkB.FL receptor degraded (targeted to the lysosomes) more quickly than TrkB.T1 (Huang et al., 2009). Moreover, TrkB.T1 regulates extracellular BDNF levels in the brain and binds, internalizes and presents BDNF to neurons via a spatial- and temporal-dependent mechanism (Biffo et al., 1995; Fryer et al., 1997; Alderson et al., 2000). Some event related with TrkB.T1 mediated sequestration and recycling of BDNF may be involved in the distinct roles of presynaptic vs. postsynaptic BDNF.

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PKC isoforms need to be phosphorylated to be active (Newton, 2003). It is well documented that PKCs play an important role in the regulation of transmitter release (West et al., 1991; Numann et al., 1994; Byrne and Kandel, 1996; Catterall, 1999; Santafé et al., 2005, 2006). Our results show that presynaptic stimulation directly increases cPKCα and cPKCβ phosphorylation (represented as via 1 in **Figure 7**), to regulate neurotransmission release. However, after muscle contraction cPKCα and cPKCβ phosphorylation is may further increased due to the increase of their synthesis (represented as via 5 in **Figure 7**). There is also functional evidence indicating that TrkB regulates ACh release via the PKC pathway (Santafé et al., 2014). Our data show that cPKCβI isoform is decisively involved in regulating ACh release induced by electrical stimulation.

Together, the data support a regulatory mechanism which has been represented in **Figure 7**. Presynaptic activity (1) directly promotes cPKC phosphorylation and (2) increases BDNF levels. BDNF acts presynaptically through (3) TrkB/PLCγ/PKC pathway to complete the activation of pPKC and thus enhance synaptic vesicle fusion and ACh release. Once PKC have executed their action, they are typically degraded thus decreasing its protein levels. The presence of contraction (2) further increases BDNF protein levels and (4) decreases TrkB.T1 protein levels, ultimately increasing the ratio TrkB.FL/T1. Even though this ratio still enhances (3) cPKC activity and its subsequent degradation, it promotes the increase in total PKC protein levels (5). Presumably, this might be due to an increase in PKC synthesis or alternatively, to a decrease in their activity-induced degradation. Thus, pPKC levels are enhanced. Consequently, presynaptic PKCs are enhanced in the nerve terminal and this establishes a larger pool of cPKC isoforms ready to promote neuromuscular transmission. This may have an impact in NMJ functionality since cPKCβI activity is required for acetylcholine release.

Several neuromuscular disorders show progressive loss of the connection between nerve and muscle. This leads to the pathological non-communication of the two tissues, eventually resulting in muscle weakness. Our results suggest that a decrease in neuromuscular activity, as it occurs in most neuromuscular disorders, could affect the BDNF/TrkB retrograde pathway linking pre- and postsynaptic components to correctly maintain neuromuscular function.

AUTHOR CONTRIBUTIONS

EH: data collection, quantitative analysis, literature search, data interpretation and statistics; VC: data collection, literature search, data interpretation and design graphic abstract; TO: electrophysiological analysis; LN, AS, MT and MMS: data collection; KH and CLJ: PCR experiments and data interpretation; JT, MAL and NG: conception and design, literature search, data interpretation and manuscript preparation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PUBLICATION 2:

Synaptic activity and muscle contraction increases PDK1 and PKCβI phosphorylation in the presynaptic membrane of the neuromuscular junction

Erica Hurtado, Víctor Cilleros, Laia Just, Anna Simó, Laura Nadal, Marta Tomàs, Neus Garcia*, Maria A. Lanuza*#, Josep Tomàs*#

Unitat d'Histologia i Neurobiologia Universitat Rovira i Virgili, Reus, Spain.

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Synaptic Activity and Muscle Contraction Increases PDK1 and PKCβI Phosphorylation in the Presynaptic Membrane of the Neuromuscular Junction

Erica Hurtado, Víctor Cilleros, Laia Just, Anna Simó, Laura Nadal, Marta Tomàs, Neus Garcia†, Maria A. Lanuza† and Josep Tomàs*†*

Unitat d'Histologia i Neurobiologia (UHNEUROB), Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain

Conventional protein kinase C βI (cPKCβI) is a conventional protein kinase C (PKC) isoform directly involved in the regulation of neurotransmitter release in the neuromuscular junction (NMJ). It is located exclusively at the nerve terminal and both synaptic activity and muscle contraction modulate its protein levels and phosphorylation. cPKCβI molecular maturation includes a series of phosphorylation steps, the first of which is mediated by phosphoinositide-dependent kinase 1 (PDK1). Here, we sought to localize PDK1 in the NMJ and investigate the hypothesis that synaptic activity and muscle contraction regulate in parallel PDK1 and cPKCβI phosphorylation in the membrane fraction. To differentiate the presynaptic and postsynaptic activities, we abolished muscle contraction with μ-conotoxin GIIIB (μ-CgTx-GIIIB) in some experiments before stimulation of the phrenic nerve (1 Hz, 30 min). Then, we analyzed total and membrane/cytosol fractions of skeletal muscle by Western blotting. Results showed that PDK1 is located exclusively in the nerve terminal of the NMJ. After nerve stimulation with and without coincident muscle contraction, total PDK1 and phosphorylated PDK1 (pPDK1) protein levels remained unaltered. However, synaptic activity specifically enhanced phosphorylation of PDK1 in the membrane, an important subcellular location for PDK1 function. This increase in pPDK1 coincides with a significant increase in the phosphorylation of its substrate cPKCβI also in the membrane fraction. Moreover, muscle contraction maintains PDK1 and pPDK1 but increases cPKCβI protein levels and its phosphorylation. Thus, even though PDK1 activity is maintained, pcPKCβI levels increase in concordance with total cPKCβI. Together, these results indicate that neuromuscular activity could induce the membrane targeting of pPDK1 in the nerve terminal of the NMJ to promote the phosphorylation of the cPKCβI, which is involved in ACh release.

Keywords: PDK1, cPKCβI, phosphorylation, neuromuscular junction, PKC, muscle contraction

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***Correspondence:**
Maria A. Lanuza
mariaangel.lanuza@urv.net
Josep Tomàs
josepmaria.tomas@urv.cat

†These authors have contributed equally to this work.

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Abbreviations: ACh, acetylcholine; AChRs, acetylcholine receptors; α-BTX, α-bungarotoxin; BDNF, Brain-derived neurotrophic factor; BSA, bovine serum albumin; DAG, diacylglycerol; GAPDH, glyceraldehyde 3-phosphatedehydrogenase; HRP, horseradish peroxidase; LAL, levator auris longus; μ-CgTx-GIIIB, μ-conotoxin GIIIB; neurotrophic factor; NMJ, neuromuscular junction; PBS, phosphate buffer saline; PDK1, phosphoinositide-dependent kinase 1; PKC, Protein kinase C; PVDF, polyvinylidene difluoride; Ser, serine; Thr, threonine; TRITC, Tetramethylrhodamine; TrkB, tyrosine receptor kinase B; TSBT, Tween 20.

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INTRODUCTION

Protein kinase C (PKC) is a common signaling node of many cellular processes, being a crucial regulator of neuronal excitability, neurotransmitter release and synaptic transmission in the nervous system (Dempsey et al., 2000; Lanuza et al., 2014; Tomàs et al., 2014). Several PKC isoforms are expressed and differently regulated in the skeletal muscle and, particularly, at the neuromuscular junction (NMJ; Hilgenberg and Miles, 1995; Lanuza et al., 2000; Perkins et al., 2001; Li et al., 2004; Besalduch et al., 2010, 2013; Obis et al., 2015a,b). Specifically, the conventional PKC βI (cPKCβI) has been involved in the regulation of diverse cellular functions including neurotransmission (Hurtado et al., 2017). It is located exclusively at the nerve terminals of NMJ and muscle contraction retrogradely enhances its levels through the brain-derived neurotrophic factor (BDNF)/tropomyosin receptor kinase B (TrkB) signaling (Hurtado et al., 2017).

PKC subcellular location is closely related with its activity. Different evidence show that PKC undergoes a process of maturation before catalytic competence (Parekh et al., 2000; Newton, 2003). In order to mature, PKC undergo a series of three phosphorylations, the first of which is mediated by phosphoinositide-dependent kinase 1 (PDK1). Membrane location confers to PKC a permissive change that enables PDK1 to access and phosphorylate its activation loop. The mature cPKCs, now "primed" for activation, are released into the cytosol and kept in an inactive conformation (Oancea and Meyer, 1998; Violin et al., 2003; Griner and Kazanietz, 2007). In the presence of intracellular calcium, diacylglycerol (DAG) and phosphatidylserine, cPKCs are tethered to the membrane ready for substrate binding, phosphorylation and the activation of downstream signaling effectors (Colón-González and Kazanietz, 2006). After their activation, PKC is downregulated through a poorly understood mechanism. In particular, the short half-life of DAG could be important for cPKC signaling termination. However, a ubiquitin–proteasome-dependent pathway for PKC isoforms has also been described (Lee et al., 1996; Lu et al., 1998; Leontieva and Black, 2004). Recent findings show that PKCs might also be present in non/hypophosphorylated forms being their phosphorylation inducible after cellular stimulation (Zhou et al., 2003; Wang et al., 2007; Osto et al., 2008; Freeley et al., 2011). Consistent with these authors, we recently found that synaptic activity enhances the phosphorylation of cPKCβI (Hurtado et al., 2017). As stated above, cPKCβI has a key role in the regulation of neurotransmission in the presynaptic component of the NMJ. Therefore, the mechanisms involved in maturation and activation of cPKCβI must be identified if the physiological functions of this isoform are to be better understood.

The discovery of PDK1 as the upstream kinase for PKCs represented an important step towards understanding PKC regulation. PDK1 is a serine (Ser)/threonine (Thr) kinase which needs to be targeted to the plasma membrane to interact with and phosphorylate its substrates such as PKC (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Balendran et al., 2000). Although the action of PDK1 on PKC signaling has been extensively studied, how PDK1 activity is regulated is still controversial and whether PDK1 is modulated by synaptic activity in the NMJ remains unknown.

In the current study, we localized PDK1 at the NMJ and we investigated the hypothesis that synaptic activity and muscle contraction regulates PDK1 and its substrate cPKCβI phosphorylation in the membrane fraction.

MATERIALS AND METHODS

Animals

"Diaphragm and levator auris longus (LAL) muscles of Sprague-Dawley rats (45–50 days; Criffa, Barcelona, Spain; RRID: RGD_5508397) were used to perform stimulation experiments, Western Blot and Immunohistochemistry. The animals were cared for in accordance with the guidelines of the European Community Council Directive for the humane treatment of laboratory animals. At least five independent animals ($n > 5$) were used to evaluate the following techniques" (Hurtado et al., 2017).

Antibodies

Primary antibodies used for Western blotting were mouse monoclonal anti-PDK1 (Cat# sc-17765 RRID: AB_626657), rabbit anti-PKCβI (Cat# sc-209 RRID: AB_2168968) and goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH); (Cat# sc-20358 RRID: AB_641101) polyclonal antibodies, purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-Na/K-ATPase antibody was purchased from Developmental Studies Hybridoma Bank. Rabbit anti-pPKCβI (Thr642; Cat# ab75657 RRID: AB_1310586) polyclonal antibody was purchased from Abcam. Rabbit anti-phosphorylated PDK1 (pPDK1; Ser241; Cat# 3061S RRID: AB_2161919) polyclonal antibody was purchased from Cell Signaling Technology.

The secondary antibodies used were donkey anti-rabbit conjugated to horseradish peroxidase (HRP) from Jackson Immunoresearch Labs (Cat# 711-035-152 RRID: AB_10015282). Rabbit anti-goat conjugated to HRP from Molecular probes (Cat# R21459 RRID: AB_11180332). Rabbit anti-mouse conjugated to HRP from Sigma (Cat# A9044 RRID: AB_258431).

To immunolabel the Schwann cell, the presynaptic component of the NMJ and the target protein PDK1 we used: rabbit polyclonal anti-S100 antibody (Cat# Z0311 RRID: AB_10013383), from Dako. Rabbit monoclonal anti-syntaxin-6 antibody (Cat# C34B2 RRID: AB_10829116), from Cell Signaling Technology. PDK1 localization was performed with the same antibody used for Western blotting (Cat#sc-17765 RRID: AB_626657). The secondary antibodies used were donkey anti-mouse or anti-rabbit conjugated to Alexa Fluor 488 and Alexa Fluor 647 from Molecular Probes (Eugene, OR, USA; Cat# A21202 RRID: AB_141607; Cat# A31573 RRID: AB_2536183). Postsynaptic nicotinic acetylcholine receptors (AChRs) were detected with α-bungarotoxin (α-BTX) conjugated to Tetramethylrhodamine (TRITC) from Molecular Probes (Eugene, OR, USA; Cat# T1175 RRID: AB_2313931).

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In Immunohistochemical and Western blot techniques, the absence of staining or bands when primary antibodies were omitted, served as a negative control. The appropriate blocking peptide was used to confirm the antibody specificity. Moreover, in double-staining protocols, one of the two primary antibodies were omitted to serve as a negative control.

Presynaptic Electrical Stimulation of Muscles

Diaphragm muscle was dissected with the phrenic nerve into two hemidiaphragms and placed in oxygenated Ringer solution (in nM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1, $NaHCO_3$ 12 and glucose 12.1 mM) continuously bubbled with 95% O_2/5% CO_2 at room temperature. One hemidiaphragm was used as the experimental condition and the other one as its control. Muscles were stimulated *ex vivo*, through their phrenic nerve at 1 Hz during 30 min by an A-M Systems 2100 isolated pulse generator (A-M System, Carlsborg, WA, USA). The main objective was to study independently the effect of synaptic transmission and the effect of the muscle cell contraction. To prevent muscle contraction, we used μ-conotoxin GIIIB (μ-CgTx-GIIIB, Alomone Labs Ltd, Israel; working solution 1.5 μM) that selectively inhibits sarcolemmal voltage-dependent sodium channels (VDSCs) without affecting synaptic ACh release (Favreau et al., 1999). Visible contractions of the diaphragm muscle indicated the successful nerve stimulation resulting in contraction. **Table 1** show the experimental design of the treatments. The protocol of electrical stimulation applied was described in Besalduch et al. (2010); Hurtado et al. (2017) and Obis et al. (2015a). Briefly "In Experiment #1, synaptic activity effects were assessed by comparing presynaptically stimulated muscles blocked by μ-CgTx-GIIIB with non-stimulated muscles also incubated with μ-CgTx-GIIIB (referred to as the *Stimulation* condition in the figures). In Experiment #2, muscle contraction *per se* was assessed by comparing stimulated/contracting muscles to presynaptically stimulated muscles blocked by μ-CgTx-GIIIB (referred to as the *Contraction* condition in the figures). In Experiment #3, to assess the complete effect of synaptic activity with the resulting muscle contraction, we compared stimulated/contracting muscles with non-stimulated muscles, without incubate with μ-CgTx-GIIIB (referred to as the *Stimulation with Contraction* condition in the figures). At least five animals were used" (Hurtado et al., 2017).

Western Blot

We obtained the samples as described in Hurtado et al. (2017). In brief, "diaphragm muscles were dissected, frozen in liquid nitrogen, and stored at −80°C before use. The muscles were homogenized using a high-speed homogenizer (overhead stirrer, VWR International, Clarksburg, MD, USA) in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail (1/100; Sigma-Aldrich, St. Louis, MO, USA). Insoluble material was removed

TABLE 1 | Summary of the electrical stimulation experiments.

Experiment	Control treatment	Treatment	Final outcome
#1 Presynaptic stimulation	**No stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIIB preincubation. 3. Incubation in Ringer solution without stimulation.	**Stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIIB preincubation. 3. Phrenic nerve stimulation with contraction blocked.	**Effect of presynaptic stimulation**
#2 Contraction	**Stimulation, blocked contraction** 1. Hemidiaphragm extraction. 2. μ-conotoxin GIIIB preincubation. 3. Phrenic nerve stimulation with contraction blocked.	**Stimulation, contraction** 1. Hemidiaphragm extraction. 2. Preincubation in Ringer solution. 3. Phrenic nerve stimulation with contraction.	**Effect of muscle contraction**
#3 Presynaptic stimulation with contraction	**No stimulation, not blocked contraction** 1. Hemidiaphragm extraction. 2. Incubation in Ringer solution without stimulation.	**Stimulation, contraction** 1. Hemidiaphragm extraction. 2. Phrenic nerve stimulation with contraction.	**Effect of presynaptic stimulation with contraction.**

The Table has been published in the original article by Hurtado et al. (2017). The original article is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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by centrifugation at 1000 *g* for 10 min at 4°C. The supernatants were collected and centrifuged at 15,000 *g* for 20 min at 4°C. Finally, the resulting supernatants (total protein lysates) were collected. Protein concentrations were determined by using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Experimental procedures were performed to determine the linear and quantitative dynamic range for each target protein and the appropriate dilutions of samples were used for accurate and normalized quantitation by means of densitometric analysis. To isolate the membrane and cytosolic fractions, diaphragm muscles were dissected and homogenized using a high-speed homogenizer in ice-cold lysis buffer without detergents (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, sodium orthovanadate 1 and protease inhibitor cocktail (1/100). The homogenized samples were cleared at 1000 g for 15 min, and the resulting supernatant was further centrifuged at 130,000 *g* for 1 h. The supernatant was the cytosolic fraction and the pellet, the membrane fraction. The pellet was resuspended in lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail (1/100). Protein concentrations were determined in the same way as total protein lysates (see above). Validation of the purity of the subcellular fractionation was determined by examining the presence of fraction-specific housekeeping proteins like GAPDH for cytosol and Na/K-ATPase for membrane by Western blotting".

Protein samples of 30 μg were separated through 8% SDS-polyacrylamide gels. After electrophoresis, the gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond^{TM}-P; Amersham, GE Healthcare) using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). For immunodetection, the membrane was blocked with Tris-buffered saline 0.1% Tween 20 (TBST) containing 5% (W/V) bovine serum albumin (BSA) for phosphorylated proteins and nonfat dry milk for non-phosphorylated proteins for an hour. Membranes were incubated with the primary antibody (specific for the interest protein) overnight at 4°C and then incubated with the corresponding secondary antibody linked to a HRP for 1 h. Finally, membranes were revealed with the Bio-Rad ECL kid and imaged with the ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, CA, USA).

Western Blot quantification between the experimental sample and the control was realized from the same blot image with the ImageJ software (ImageJ, RRID: SCR_003070). GAPDH and Na/K-ATPase proteins were used as loading controls, as well as total protein staining (Sypro Ruby protein blot stain, Invitrogen). The quantification values were normalized to: (1) the background and to (2) total protein quantification. Data are mean values ± SEM. Statistical significance of the difference between groups was evaluated under the Wilcoxon test or the Student's *t*-test and the normality of the distributions was tested with the Shapiro-Wilk test. The criterion for statistical significance was $p < 0.05$ vs. the control (*) and at least five animals were evaluated in any condition.

Immunohistochemistry and Confocal Microscopy

To localize PDK1 at the NMJ we performed immunohistochemistry in LAL muscle and diaphragm. Muscles were fixed for 30 min using 4% paraformaldehyde, then rinsed with phosphate buffer saline (PBS) and incubated in 0.1 M glycine in PBS. Then, muscles where incubated with goat serum overnight at 4°C, rinsed with PBS, and then incubated with 1% Triton X-100/4% BSA in PBS overnight at 4°C. Incubation with the primary antibodies, was done overnight at 4°C (anti PDK1; anti syntaxin to label the axon terminal; anti-S100 to label Schwann cells) and then rinsed with PBS. Finally, muscles were incubated in a mixture of appropriate secondary antibodies, overnight at 4°C. To detect AChRs we used α-BTX conjugated with TRITC. The appropriate negative controls were done in at least three muscles as described above. Moreover, there was not cross over between antibodies. For imaging, a laser-scanning confocal microscope (Nikon TE2000-E) was used and images were assembled using Adobe Photoshop software without modifying the contrast or brightness (Adobe Systems, San Jose, CA, USA; RRID: SCR_014199). Care was taken to the possible contamination of one channel by another. For negative controls imaging, the photomultiplier tube gains and black levels were not modified. At least 25 endplates per muscle were observed, and at least five muscles were studied.

RESULTS

PDK1 in the Skeletal Muscle

Western blot analysis of PDK1 was carried out to determine its presence in the skeletal muscle. The anti-PDK1 antibody was raised against a peptide corresponding to the residues 229–556 of PDK1. This antibody revealed a major band of the predicted molecular weight (68 kDa), suggesting the monospecificity of the antibody (**Figure 1**). Phosphorylation of PDK1 was analyzed using an antibody raised against a peptide corresponding to the residues surrounding the Ser241 of human PDK1, a region identical to the rat PDK1 (Uniprot sequences O15530 and O55173, respectively). This antibody reacted with a unique band that is consistent with the PDK1 predicted molecular weight (**Figure 1**). Western blotting results revealed significant amounts of PDK1 and pPDK1 in the skeletal muscle in basal conditions (**Figure 1**). Subsequently, we sought to identify the cellular distribution of PDK1 at the NMJ by immunofluorescence.

Localization of PDK1 in the Nerve Terminals of the NMJ

The localization of PDK1 in the NMJ is essential to elucidate its function. Therefore, immunofluorescence coupled with confocal microscopy was carried out to stain PDK1 and the three cellular elements of the NMJ ($n = 5$; 25–30 endplates per muscle). Images in **Figure 2** show intense immunoreactivity for PDK1 (in green) in the synaptic area identified with AChR labeling (in red). **Figures 2A,B** (cross-view confocal section) show a double

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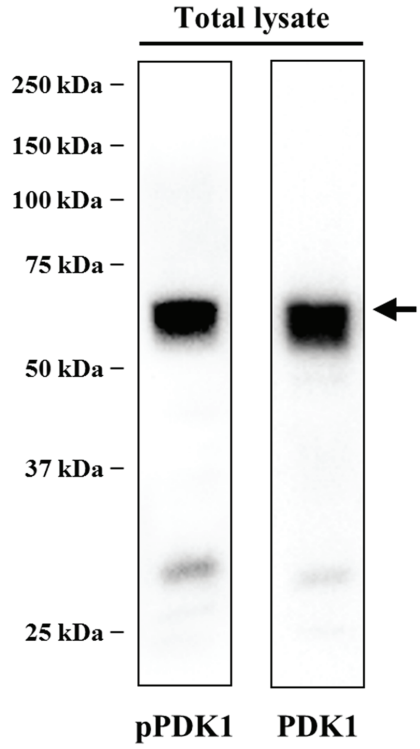

FIGURE 1 | PDK1 is expressed in skeletal muscle in basal conditions. Determination of the specificity of anti-phosphorylated PDK1 (pPDK1) S241 antibody (CS-3061) and anti-PDK1 antibody (sc-17765) by immunoblotting. Thirty microgram of protein from total lysate were size fractionated by SDS-PAGE on 8% acrylamide minigels and transferred to polyvinylidene difluoride (PVDF) membranes. The antibodies used only recognized the corresponding protein, reacting with a band consistent with its predicted molecular weight. Western blot analysis revealed significant amounts of PDK1 and pPDK1 in the skeletal muscle in basal conditions. Abbreviations: PDK1, phosphoinositide-dependent kinase 1; pPDK1, phosphorylated phosphoinositide-dependent kinase 1.

labeled NMJ: AChRs (marked with fluorescently labeled α-BTX, in red) and PDK1 in green. These figures show PDK1-positive green immunolabeling concentrated at the presynaptic position over the red postsynaptic gutters, without immunoreactivity for muscle cells. Moreover, the pre-terminal axon was also PDK1-positive.

We also performed a triple staining in which we co-localized PDK1 (in green), muscle cell (AChR, in red), nerve terminal (labeled with syntaxin, in blue) and/or Schwann cells (labeled with S100, in blue; **Figures 2C,D**). The fine granulated label for PDK1 colocalized with syntaxin in the presynaptic nerve terminal position (over the AChRs-positive postsynaptic red gutters). The inset in **Figure 2C** shows a good colocalization between PDK1 and syntaxin indicating the presynaptic localization of PDK1 in the nerve terminal of the NMJ. Moreover, PDK1 was not colocalized with the Schwann cell (**Figure 2D**, see arrows). Altogether, these results indicate that PDK1 is exclusively located at the presynaptic component of NMJ.

Total PDK1 Levels and Its Phosphorylation Are Unaltered after Synaptic Activity and Muscle Contraction

PDK1 is an upstream regulator of numerous protein kinases of the AGC kinase superfamily, including conventional PKC isoforms (Dutil et al., 1998). Previous results showed that pre- and postsynaptic neuromuscular activities regulate specifically cPKCβI protein levels (Besalduch et al., 2010) and its phosphorylation (Hurtado et al., 2017) in skeletal muscle total lysates. Therefore, our first objective was to determine whether synaptic activity and/or muscle contraction modulate PDK1 and its phosphorylation (pPDK1) in the skeletal muscle. In our *in vivo* experimental system, we can distinguish the effects of synaptic activity from those of muscle contraction. As described in Hurtado et al. (2017) "Synaptic activity includes the presynaptic events related with nerve stimulation (1 Hz, 30 min), synaptic transmission and endplate potential generation due to ACh signaling (referred to as the *Stimulation* condition in the figures). Muscle contraction includes membrane depolarization of the muscle fiber involving voltage-dependent sodium channels and the resulting myofiber contraction (referred to as the *Contraction* condition in the figures). Finally, presynaptic *Stimulation with Contraction* treatment includes the effects of synaptic activity and muscle contraction, showing complete neuromuscular activity".

We analyzed by Western blotting how activity affects the level of PDK1 and its phosphorylation (pPDK1) in total lysates. Results revealed that PDK1 and pPDK1 levels, as well as pPDK1/PDK1 ratio, remained unaltered after nerve stimulation with and without coincident muscle contraction (Stimulation $n = 7$, Contraction $n = 6$ and Stimulation with Contraction $n = 6$; **Figure 3**). This indicates that there is a stable pool of PDK1 at the NMJ catalytically competent during synaptic activity. However, although neuromuscular activity does not affect total PDK1 levels nor its phosphorylation, we recently determined that it induces the phosphorylation of its target cPKCβI. Therefore, PDK1 activation might be promoted by neuromuscular activity through PDK1 translocation to the plasma membrane.

Synaptic Activity Increases Phosphorylated PDK1 and PCKβI in the Membrane Fraction of Skeletal Muscle

Several lines of evidence show that PDK1 targeting to the plasma membrane is determinant for its activation (Yang et al., 2002a,b), leading to the phosphorylation of PKC, as it is also located in the plasma membrane. Thus, we proceeded to analyze how synaptic activity and/or muscle contraction modulate PDK1 and cPKCβI protein levels and their phosphorylation in the cytosol and membrane fractions. The purity of membrane and cytosol fractionation was confirmed by immunoblotting of Na/K-ATPase and GAPDH as specific protein markers. Results showed

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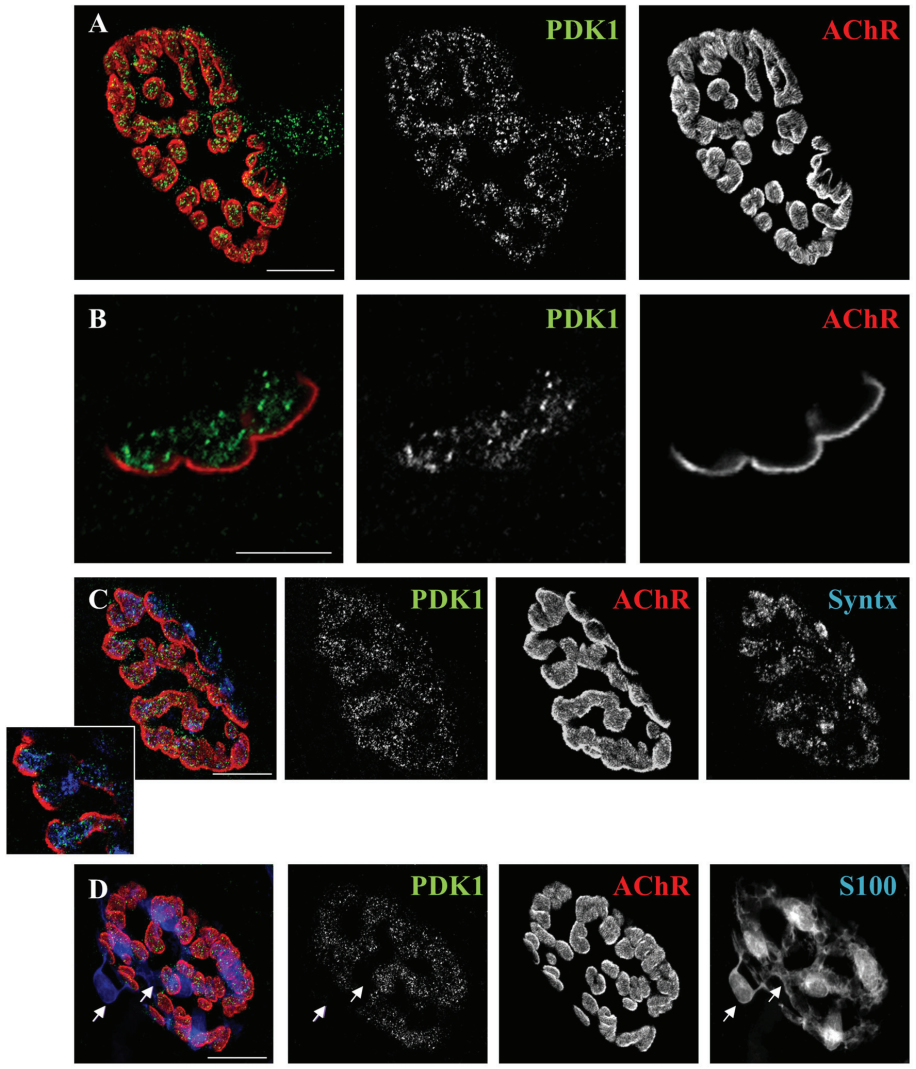

FIGURE 2 | PDK1 is localized in the nerve terminals of the neuromuscular junction (NMJ). **(A,B)** Double staining labeled PDK1 (in green) and AChRs (fluorescent α-BTX in red). The images show PDK1-positive green immunolabeling concentrated at the presynaptic area over the red postsynaptic gutters. **(C)** Triple staining labeled PDK1 (in green), AChRs (in red) and nerve terminal (with anti-syntaxin antibody in blue, Syntx). The fine granulated label for PDK1 was well colocalized with syntaxin in the presynaptic nerve terminal position (over the AChRs-positive postsynaptic red gutters). **(D)** Triple staining labeled PDK1 (in green), AChRs (in red) and Schwann cell (with anti-S100 antibody in blue) showed that PDK1 was not colocalized with Schwann cell (see arrows). Thus, indicating that PDK1 is exclusively located at the presynaptic component of NMJ (n = 5; 25–30 endplates per muscle). The scale bars indicate 10 μm. Abbreviations: PDK1, phosphoinositide-dependent kinase 1; AChRs, Acetylcholine receptors; Syntx, syntaxin; S100, S100 protein; α-BTX, α-bungarotoxin.

that the cytosolic protein GAPDH was in the cytosol fraction and essentially undetectable in the membrane fraction. As expected, the Na/K-ATPase was highly enriched in the membrane component, and undetectable in the cytosol fraction. Keranen et al. (1995) determined that only 50% of PKC species retain the PDK1-induced phosphate in their activation loop, being mature

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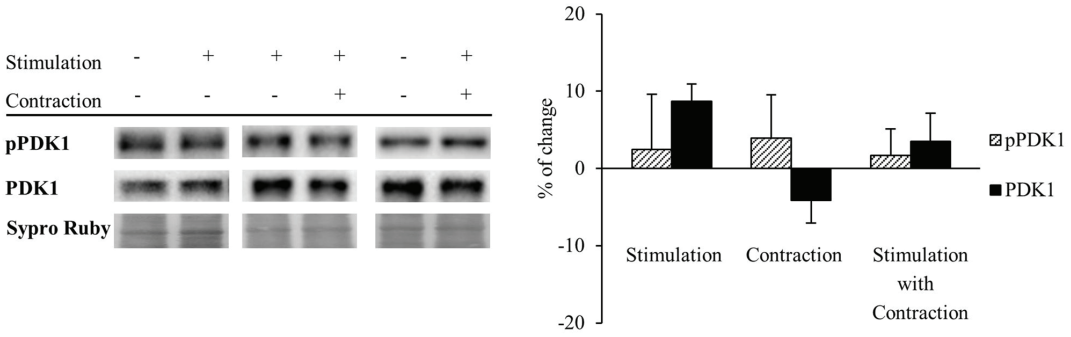

FIGURE 3 | Total PDK1 levels and its phosphorylation are unaltered after synaptic activity and muscle contraction. PDK1 and pPDK1 in presynaptic stimulation treatment, Contraction treatment and Presynaptic stimulation with contraction treatment at 1 Hz stimulation for 30 min. Presynaptic stimulation has been simplified as *Stimulation*. Each column has been compared to its respective control (see **Table 1**). Results showed that PDK1 and pPDK1 levels, as well as pPDK1/PDK1 ratio, remained unaltered after nerve stimulation with and without muscle contraction. Data are mean percentage ± SEM, (Stimulation $n = 7$, Contraction $n = 6$ and Stimulation with Contraction $n = 6$). Abbreviations: PDK1, phosphoinositide-dependent kinase 1; pPDK1, phosphorylated phosphoinositide-dependent kinase 1.

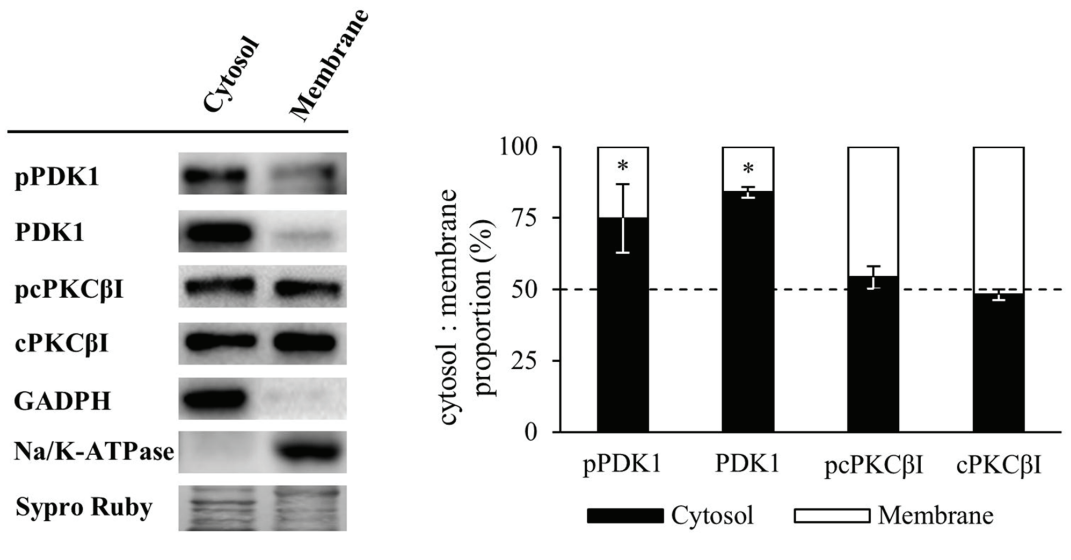

FIGURE 4 | Membrane and cytosol distribution of PDK1 and cPKCβI in basal conditions. Western Blot analysis of the distribution of PDK1 and cPKCβI in membrane and cytosol fraction of skeletal muscle. Results showed that in basal conditions, pPDK1 and PDK1 were found predominantly in the cytosol fraction while pcPKCβI and cPKCβI were present similarly in both cytosol and membrane fractions. Moreover, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was found in the cytosol fraction and essentially undetectable in the membrane fraction. As expected, the membrane protein Na/K-ATPase was highly enriched in this cellular component, and undetectable in the cytosol fraction. Data are mean percentage ± SEM, $^*p < 0.05$ ($n = 5$). Abbreviations: phosphoinositide-dependent kinase 1; pPDK1, phosphorylated phosphoinositide-dependent kinase 1; cPKCβI, conventional protein kinase C βI; pPKCβI, phosphorylated conventional protein kinase C βI.

cPKCs quantitatively autophosphorylated at their turn-motif and hydrophobic loop. Therefore, to avoid the interference of dephosphorylation, we analyzed the phosphorylation of cPKCβI with an antibody against Thr642 turn-motif phosphorylation, which is the subsequent phosphorylation induced by PDK1 and it is required for kinase activity (Zhang et al., 1994). Our results showed that, in basal conditions, pPDK1 and PDK1 were found predominantly in the cytosol fraction (cytosol:membrane, pPDK1: 74.95:25.05% ± 12.02, $p < 0.05$; PDK1: 84.00:16.00% ± 1.84, $p < 0.05$; $n = 5$) while pcPKCβI and cPKCβI were present similarly in both cytosol and membrane fractions (cytosol:membrane, pcPKCβI: 54.21:45.79% ± 3.78,

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$p > 0.05$; cPKCβI: 48.28:51.72% ± 1.88; $p > 0.05$; $n = 5$; **Figure 4**).

Next, we determined how synaptic activity without contraction affects the levels and the phosphorylation of PDK1 and cPKCβI in the cytosol and membrane fractions ($n = 5$; **Figure 5**). Results showed that synaptic activity does not affect significantly the level of any considered protein in the cytosol although the levels of PDK1, pPDK1 and pcPKCβI tended to decrease. Therefore, the ratios pPDK1/PDK1 and pcPKCβI/cPKCβI remained unchanged. However, synaptic activity significantly increased both pPDK1 and its substrate, pcPKCβI in the membrane (pPDK1: 37.31% ± 4.75, $p < 0.05$; pcPKCβI: 26.11% ± 4.15, $p < 0.05$). In addition, total protein levels of PDK1 were maintained and cPKCβI were significantly decreased (cPKCβI: −72.73% ± 3.12, $p < 0.05$; **Figure 5**). Thus, the increase in both pPDK1/PDK1 and pcPKCβI/cPKCβI ratios (35.88% ± 0.59, $p < 0.05$ and 362.95% ± 3.44, $p < 0.05$; respectively) indicate that synaptic activity enhances phosphorylation of PDK1 and cPKCβI. Together, these results show that presynaptic activity increases the levels of pPDK1 in the membrane fraction, a subcellular location known to be important for PDK1 function. Because this increase of pPDK1 coincides with a significant increase of pcPKCβI in the membrane fraction, this might indicate that synaptic activity increases PDK1 function to phosphorylate cPKCβI.

Muscle Contraction Maintains PDK1 and pPDK1 Levels but Increases cPKCβI and pcPKCβI Levels in the Membrane Fraction of Skeletal Muscle

Because muscle activity *per se* has a critical role to enhance presynaptic cPKCβI (Besalduch et al., 2010; Hurtado et al., 2017), we analyzed the role of muscle contraction over PDK1 and cPKCβI protein levels and their phosphorylation in the cytosolic and membrane fraction ($n = 5$; **Figure 6**). We observed that muscle contraction increased cPKCβI protein levels in the cytosolic fraction (cPKCβI: 41.22% ± 10.29, $p < 0.05$) without altering pPDK1, PDK1 and pcPKCβI levels. Thus, the ratio pPDK1/PDK1 was maintained while pcPKCβI/cPKCβI decreased due to the increase of the total cPKCβI levels (−43.09% ± 1.62, $p < 0.05$). So even though PKCβI is increased, muscle contraction does not promote its phosphorylation in the cytosol. Regarding the membrane fraction, pPDK1 and PDK1 levels did not change after contraction but both pcPKCβI and cPKCβI were significantly increased (pcPKCβI: 38.19% ± 4.35 $p < 0.05$; cPKCβI: 37.23% ± 3.50, $p < 0.05$). Thus, the ratio pcPKCβI/cPKCβI in the membrane fraction remained the same indicating that muscle contraction enhances phosphorylation of cPKCs due to an increase of total protein PKC protein level.

These results together suggest that muscle contraction might induce the synthesis of cPKCβI, increasing total protein levels in both cytosol and membrane fractions. The increased amount of pcPKCβI in the membrane might be explained by an increase of total PKC protein level, as PDK1 activity is maintained (see **Figure 6**).

To reinforce the previous results, we assessed the complete neuromuscular activity (synaptic activity with muscle contraction; $n = 5$). In the membrane fraction, pPDK1 was increased (achieved by synaptic activity; pPDK1: 40.77% ± 9.27, $p < 0.05$) but without altered PDK1 protein levels (PDK1: 11.37% ± 1.58, $p > 0.05$). Moreover, total cPKCβI and pcPKCβI levels were also increased in the membrane fraction due to muscle contraction (cPKCβI: 31.88% ± 8.59, $p < 0.05$; pcPKCβI: 30.42% ± 8.19, $p < 0.05$).

Altogether, these results suggest that synaptic activity induces the phosphorylation of cPKCβI through the translocation of pPDK1 to the membrane. Furthermore, contraction increases the synthesis of cPKCβI and consequently the amount of pcPKCβI even maintaining PDK activity.

DISCUSSION

PDK1 is a crucial Ser/Thr kinase which activates as many as 23 protein kinases of the AGC family, including PKC, by phosphorylating their T-loop sites (Toker, 2003; Mora et al., 2004; Bayascas, 2010; Pearce et al., 2010). Although the importance of PDK1 in PKC signaling has been well characterized (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Balendran et al., 2000), its synaptic localization and function in the nervous system has not been fully determined. Thus, in this study, we localized PDK1 at the neuromuscular synapse and we investigated the hypothesis that synaptic activity and muscle contraction regulates PDK1 and cPKCβI phosphorylation in the membrane fraction. Our results support that PDK1 is localized in the nerve terminals of the NMJ. Moreover, synaptic activity increases pPDK1 levels in the membrane. Because the increase of pPDK1 coincides with a significant increase of pcPKCβI in the membrane fraction, this might indicate that synaptic activity increases PDK1 function to phosphorylate cPKCβI. Furthermore, when contraction is present, the total amount of cPKCβI is increased in both cytosol and membrane fraction, suggesting an activation of its synthesis.

Synaptic Activity Increases Phosphorylated PDK1 and pcPKCβI in the Membrane Fraction of the Skeletal Muscle

In the skeletal muscle, PDK1 is mainly present in the cytosolic fraction in basal conditions and the confocal microscopy shows that it is only expressed in the nerve terminal of the rat NMJ. Consistent with that, PDK1 has been located also in the nerve terminals at the *Drosophila* NMJ (Cheng et al., 2011). It is surprising that while several PKC isoforms are located in the different cells of the rat NMJ (Perkins et al., 2001; Besalduch et al., 2010, 2013; Lanuza et al., 2014; Obis et al., 2015a), PDK1 is located exclusively in the nerve terminal. This fact may be related with a specific role of this protein in priming presynaptic kinases (such nPKCε and cPKCβI) selectively involved in the rapid and complex exocytotic process of transmitter release. Due to its presynaptic location, PDK1 activation could be susceptible to synaptic activity influence. Different studies

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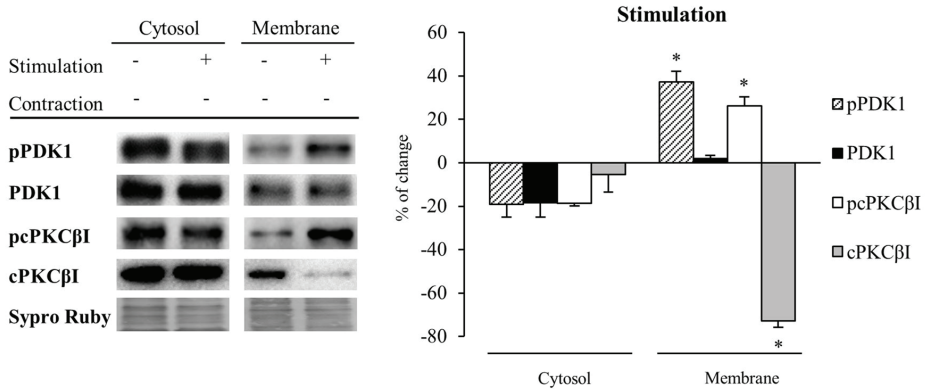

FIGURE 5 | Synaptic activity increases pPDK1 and PCKβI in the membrane fraction of skeletal muscle. Western Blot of PDK1 and pPDK1 after isolation of membrane and cytosol fractions in presynaptic stimulation treatment at 1 Hz stimulation for 30 min. Presynaptic stimulation has been simplified as *Stimulation*. Each column has been compared to its respective control (see **Table 1**). Results showed that synaptic activity does not affect significantly the level of any protein in the cytosol. Therefore, the ratios pcPKCβI/cPKCβI and pPDK1/PDK1 remain the same. However, both pPDK1 and its substrate, pcPKCβI were significantly increased in the membrane fraction. Thus, both ratios pcPKCβI/cPKCβI and pPDK1/PDK1 were increased indicating that synaptic activity specifically enhances phosphorylation of PDK1 and cPKCβI. Data are mean percentage ± SEM, $*p < 0.05$ ($n = 5$). Abbreviations: phosphoinositide-dependent kinase 1; pPDK1, phosphorylated phosphoinositide-dependent kinase 1; cPKCβI, conventional protein kinase C βI; pPKCβI, phosphorylated conventional protein kinase C βI.

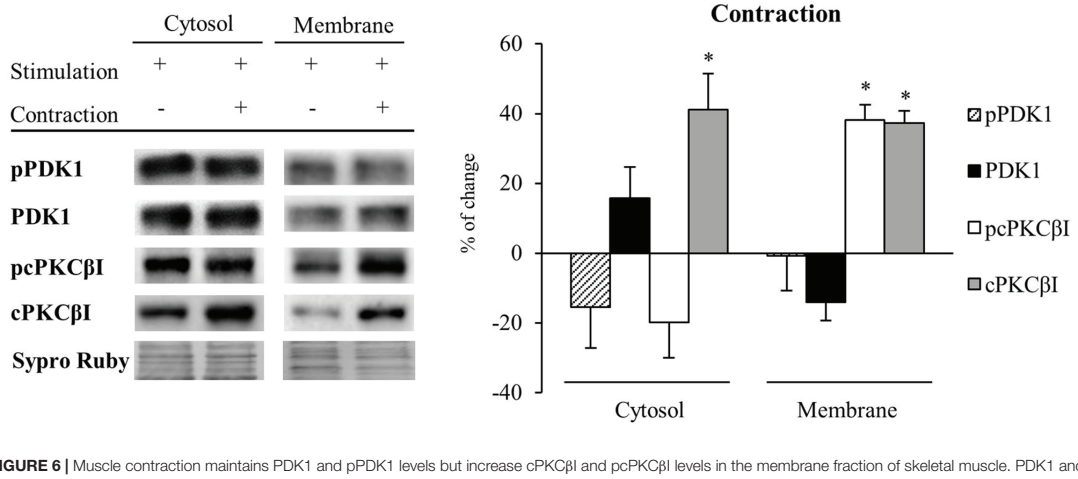

FIGURE 6 | Muscle contraction maintains PDK1 and pPDK1 levels but increase cPKCβI and pcPKCβI levels in the membrane fraction of skeletal muscle. PDK1 and pPDK1 after isolation of membrane and cytosol fractions in Contraction treatment at 1 Hz stimulation for 30 min. Each column has been compared to its respective control (see **Table 1**). We observed that muscle contraction increased cPKCβI protein levels in the cytosolic fraction but pPDK1, PDK1 and pcPKCβI levels remained the same. Thus, the ratio pPDK1/PDK1 was maintained while pcPKCβI/cPKCβI decreased due to the increase of the total protein. Moreover, in the plasma membrane, pPDK1 and PDK1 were also not modified but pcPKCβI and cPKCβI were significantly increased. Thus, the ratio pPDK1/PDK1 and pcPKCβI/cPKCβI in the membrane fractions remained the same. Data are mean percentage ± SEM, $*p < 0.05$ ($n = 5$). Abbreviations: phosphoinositide-dependent kinase 1; pPDK1, phosphorylated phosphoinositide-dependent kinase 1; cPKCβI, conventional protein kinase C βI; pPKCβI, phosphorylated conventional protein kinase C βI.

have shown that PDK1 is constitutively phosphorylated on at least five serine residues (S25, S241, S393, S396 and S410; Casamayor et al., 1999). However, other studies suggest that signaling pathways activated by insulin-like growth factor 1 (IGF-1) can further increase the degree of PDK1 phosphorylation on these sites (Scheid et al., 2005). Our results suggest that PDK1 is constitutively phosphorylated in the S241 site after synaptic activity and muscle contraction in total skeletal muscle lysates. However, we demonstrated that the subcellular localization of pPDK1 is inducible by synaptic activity. Activity is able to translocate pPDK1 to the plasma membrane where PDK1 is in the optimal situation to interact with and

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phosphorylate its substrates (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Balendran et al., 2000; Yang et al., 2002a,b). It should be noted that pPDK1 is slightly, but not significantly, reduced in the cytosol fraction and it may be because PDK1 is mainly present in the cytosol fraction. Thus, small decreases in their protein levels might not be significantly appreciated, but enough to detect a significant increase in the membrane fraction. PI3-kinase (PI3K) activity recruits PDK1 to membranes, leading to phosphorylation of downstream substrates (Alessi et al., 1997). Here we show that this recruitment to membrane is promoted by the synaptic activity at the NMJ and this mechanism may be Ca^{2+} dependent. PDK1 with its PH domain binds to either PIP3 or PIP2 and is translocated to the plasma membrane. Evidence show that PDK1 does not have any domain that directly interacts with calcium. However, recent evidence shows that in central nerve terminals an increase of intracellular calcium promotes PI3K activity by an unknown calcium sensor (Nicholson-Fish et al., 2016). Therefore, calcium influx may increase PIP3 production (by enhancing PI3K) which, in turn, could promote PDK1 translocation to the membrane. It has been evidenced that PDK1 is the upstream kinase which directly phosphorylates the activation loop of PKC isoforms (Dutil and Newton, 2000). Although PDK1 is constitutively active (Casamayor et al., 1999) the translocation to the membrane induced by synaptic activity may provide an important mechanism for prolonged activation of PKCs.

PKC family has emerged as essential for the control of aspects of higher-level signal organization. It is a multigene family of Ser/Thr kinases that comprises ∼2% of the human kinome. In the nervous system, synaptic transmission (Dempsey et al., 2000; Lanuza et al., 2007; Tomàs et al., 2014) is decisively modulated by the involvement of several PKC isoforms differently localized and regulated (Hilgenberg and Miles, 1995; Lanuza et al., 2000; Perkins et al., 2001; Li et al., 2004; Besalduch et al., 2010, 2013; Obis et al., 2015a,b). For instance, the novel nPKCθ has several roles which include the neuromuscular system development (Li et al., 2004; Lanuza et al., 2006, 2010; Besalduch et al., 2011) and differentiation and homeostasis of the skeletal muscle (Tokugawa et al., 2009; Madaro et al., 2011, 2012). nPKCθ may regulate excitability and muscle contraction through the modulation of chloride channel activity (Camerino et al., 2014). In addition, the novel nPKCε coupling is clearly involved to maintain or potentiate ACh release in the NMJ (Obis et al., 2015b). Interestingly, conventional cPKCβI is exclusively located in the presynaptic component, is modulated by both synaptic activity and muscle contraction and, in turn, is directly involved in transmitter release in the NMJ (Besalduch et al., 2010; Hurtado et al., 2017). It is interesting to note that PDK1, as well as cPKCβI, is exclusively located in the nerve terminal at the NMJ.

To become competent and able to respond to second messengers, PKCs undergo a previous process of maturation (Parekh et al., 2000; Newton, 2003) and its activation requires translocation of the enzyme to membrane (Kraft et al., 1982). Conventional cPKC maturation involves three phosphorylation steps at specific sites, the first of which is mediated by PDK1 in the catalytic domain activation loop. In contrast, the two carboxy-terminal phosphorylations in the turn and hydrophobic motifs have been shown to undergo autophosphorylation events subsequent to the PDK1 mediated phosphorylation (Cazaubon and Parker, 1993; Keranen et al., 1995; Dutil et al., 1998). Membrane location confers to PKC a permissive change that promotes activation loop phosphorylation by PDK1. Mature cPKCs, are released into the cytosol and kept in an inactive conformation ready to be activated (Oancea and Meyer, 1998; Violin et al., 2003; Griner and Kazanietz, 2007). However, recent findings show that PKCs can also exist in non/hypophosphorylated forms, with cellular stimulation resulting in inducible phosphorylation and activation (Zhou et al., 2003; Wang et al., 2007; Osto et al., 2008). Obis et al. (2015a) described that synaptic activity modulates phosphorylation of nPKCε at the NMJ. Moreover, presynaptic cPKCβI phosphorylation is enhanced by synaptic activity and muscle contraction (Hurtado et al., 2017). Here, our results showed that phosphorylation of cPKCβI is inducible by synaptic activity and specifically increased in the plasma membrane. Thus, at the membrane fraction, the significant increase of pPDK1 described above, coincides with a significant increase of pcPKCβI. Because PDK1 directly interacts with cPKCβI, among other PKC isoforms, through the kinase domain of the enzyme (Dutil et al., 1998; Le Good et al., 1998), this might indicate that synaptic activity increases PDK1 function to phosphorylate cPKCβI allowing for its substrate binding, phosphorylation and the activation of downstream signaling effectors. The increase of pcPKCβI in the membrane after synaptic activity is accompanied by a significant decrease of the total cPKCβI indicating the described downregulation of the PKC after activation. This result was also previously demonstrated (Hurtado et al., 2017) and here we specifically found that is in the membrane where the downregulation occurs. Furthermore, total but not phosphorylated cPKCβI protein levels depends on synaptic activity-induced BDNF/TrkB signaling at the NMJ (Hurtado et al., 2017) indicating that PDK1 activity phosphorylating cPKCβI would be not modulated by the BDNF/TrkB signaling pathway.

Muscle Contraction Maintains PDK1 and pPDK1 Levels but Increases cPKCβI and pcPKCβI Levels in the Membrane Fraction of Skeletal Muscle

PDK1 has been related with cell contraction and cell migration. Some studies suggest that the kinase activity of PDK1 was not required for the regulation of cortical subplasmalemic actin or cell contraction (Pinner and Sahai, 2008); this contrasts with previous reports suggesting that PDK1 regulates actin organization through PKB/Akt, PAK or integrinβ3 (Lim et al., 2004; Weber et al., 2004; Xie et al., 2006; Primo et al., 2007). However, whether PDK1 activity is related with muscle contraction *in vivo* in the skeletal muscle is still unknown. Here we show that muscle contraction does not modify pPDK1 and PDK1 levels either in the cytosol or the membrane fraction,

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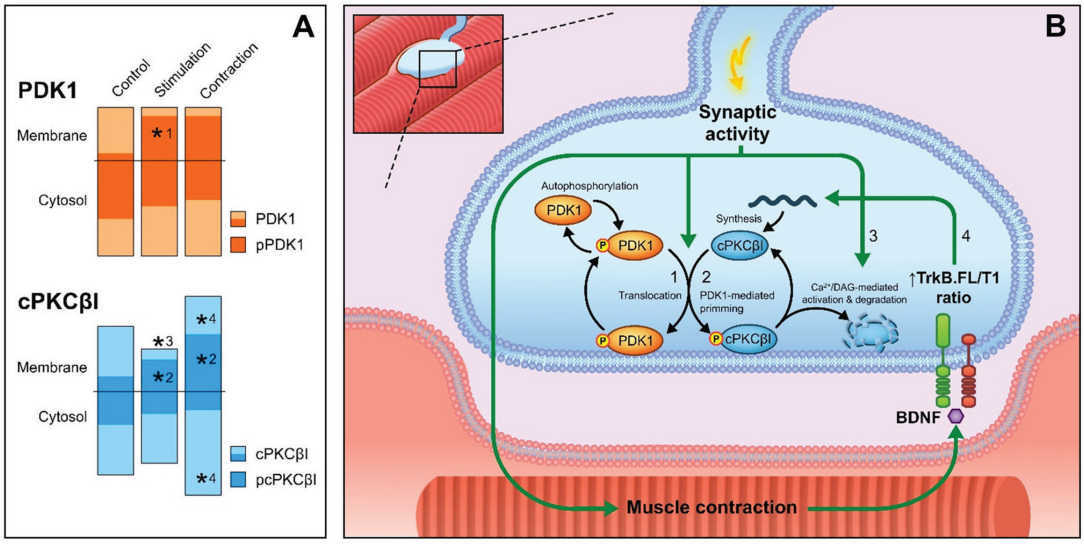

FIGURE 7 | Overview of PDK1 function and cPKCβI phosphorylation at the NMJ. **(A)** Summary of the results of this work. Columns represent the total protein levels of PDK1 (in orange), pPDK1 (in dark orange), cPKCβI (in blue) and pcPKCβI (in dark blue) in the membrane and cytosol compartments (indicated with a horizontal line) $^*p < 0.05$. **(B)** Proposed model of the action of PDK1 on cPKCβI phosphorylation at the NMJ. Total PDK1 protein and phosphorylation levels are maintained throughout all conditions. (#1) Synaptic activity induces the translocation of pPDK1 to the membrane. Consistent with the increased pPDK1 in the membrane, synaptic activity also (#2) increases the phosphorylation of cPKCβI in the same compartment. Once catalytically competent, pcPKCβI activation through synaptic activity (#3) reduces the total amount of cPKCβI in the membrane due to an increase in its activation-induced degradation. Muscle contraction (#4) increases the total amount of cPKCβI in both cytosol and membrane, suggesting an activation of its synthesis. In previous work, we determined that this enhancement is retrogradely regulated through BDNF/TrkB signaling (Hurtado et al., 2017). After muscle contraction, PDK1 activity remains unaltered and, therefore, pcPKCβI levels increase in concordance with total cPKCβI (#2). Abbreviations: phosphoinositide-dependent kinase 1; pPDK1, phosphorylated phosphoinositide-dependent kinase 1; cPKCβI, conventional protein kinase C βI; pPKCβI, phosphorylated conventional protein kinase C βI; BDNF, brain-derived neurotrophic factor; TrkB.FL, tropomyosin-related kinase B full-length, TrkB.T1, tropomyosin-related kinase B truncated isoform 1.

suggesting that its activity is mainly determined only by presynaptic activity. This result is consistent with the exclusive location of the PDK1 that we found, in the nerve terminal of the NMJ and suggests that it is not retrogradely regulated by the muscular activity.

However, PKC isoforms are differently regulated in the skeletal muscle (Hilgenberg and Miles, 1995; Lanuza et al., 2000; Perkins et al., 2001; Li et al., 2004; Besalduch et al., 2010, 2013; Obis et al., 2015a,b). Especially, our results show that conventional cPKCβI is modulated by muscle contraction in both cytosol and membrane fractions, as previously demonstrated (Besalduch et al., 2010; Hurtado et al., 2017). Specifically, we observed that muscle contraction increased cPKCβI protein levels in the cytosolic fraction suggesting that it is thus promoting its synthesis or alternatively decreasing its degradation. In addition, in the plasma membrane both pcPKCβI and cPKCβI were significantly increased suggesting that PKC synthesis, its translocation to the membrane and its phosphorylation are enhanced after muscle contraction. Thus, these results indicate that muscle contraction induces the synthesis of cPKCβI, increasing total protein levels in both cytosol and membrane fractions. Furthermore, it has been shown that presynaptic cPKCβI levels are enhanced by muscle contraction through the BDNF/TrkB signaling suggesting a retrograde regulation of this isoform (Hurtado et al., 2017). However, even though pPDK1 activity is maintained, pcPKCβI is enhanced due to the increased amount of total cPKCβI caused by muscle contraction.

Figure 7A summarizes our results. Thus, this study demonstrates that PDK1 is exclusively located in the nerve terminal of the NMJ and that synaptic activity enhances the location of its phosphorylated form in the membrane (**Figure 7**-#1), the optimal place to be active. This increment of the levels of pPDK1 in the membrane coincides with increases of its substrate pcPKCβI in the membrane (**Figure 7**-#2), suggesting that synaptic activity increases PDK1 function to phosphorylate cPKCβI. Synaptic activity reduces the total amount of cPKCβI in the membrane due to an increase in its activation-induced degradation (**Figure 7**-#3). The resulting muscle contraction may play a retrograde control over presynaptic cPKCβI to activate its synthesis (or alternatively decreasing its degradation), thus increasing the amount of cPKCβI (**Figure 7**-#4, Hurtado et al., 2017). This might explain the increase in pcPKCβI (**Figure 7**-#2). The diagram in **Figure 7B** shows this proposed mechanism of the action of PDK1 on cPKCβI phosphorylation at the

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NMJ. Importantly, pcPKCβI has a critical role in regulating transmitter release (Hurtado et al., 2017). Thus, both pre- and postsynaptic activities are needed to modulate PDK1 and cPKCβI function, ensuring an accurate neurotransmission process.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

EH: data collection, quantitative analysis, literature search, data interpretation, statistics; VC: data collection, literature search, data interpretation, design graphic abstract; LJ, LN, AS and MT: data collection; JT, MAL and NG: conception and design, literature search, data interpretation, manuscript preparation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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D. RESULTS

Chapter II

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RESULTS

Diverse evidence shows that BDNF and its receptor are involved in neurodegenerative disease as ALS. BDNF has strong pro-survival effects in developing and injured motoneurons (Mantilla et al., 2013; Peng et al., 2003) and these effects are mediated by its binding to TrkB receptors and $p75^{NTR}$. Moreover, there is a complex BDNF signalling output balanced by the negative regulation of TrkB.T1 over TrkB.FL (Dorsey et al., 2011). As BDNF signalling has been shown to be activity dependent, physical exercise has been proposed to provide benefits in ALS disease, but with contradictory results.

1. Swimming training efficiently prevented the ALS-induced increase of mBDNF levels in the skeletal muscle of ALS mice

To investigate how the BDNF/TrkB signalling pathway is affected in the skeletal muscle of ALS mice (SOD1-G93A), we first analysed the mature BDNF (mBDNF) and proBDNF proteins levels in WT (Control) and ALS mice. As in Chapter I, we used the anti-BDNF antibody, raised against a peptide sequence corresponding to the amino acids 130–247 of BDNF, a region present in both proBDNF (32 kDa) and mBDNF (14 kDa) (Zheng et al., 2010). Western blot results showed that mBDNF protein level was significantly increased in ALS mice (3.01 ± 0.29; $p<0.001$) without affecting proBDNF levels (Figure 16).

It is known that the increase of BDNF due to physical exercise has beneficial effects in the central nervous system (Cuppini et al., 2007; Zoladz and Pilc, 2010). To know whether physical exercise can modulate BDNF levels in skeletal muscles, we next compared the impact of two physical exercises (running and swimming) in ALS mice. Western blot results showed that proBDNF levels were maintained after both exercises (Figure 16). However, after the running-based training, mBDNF levels decreased but were still increased compared to control (1.82 ± 0.21; $p<0.001$) while

the swimming-based training was able to maintain the mBNDF levels close to the control (1.06 ± 0.06; p>0.05).

Therefore, ALS disease increase the mBDNF and, both exercises significantly reversed the ALS-induced increase of mBDNF levels but only the swimming-based training efficiently preserved the mBDNF levels in ALS mice close to the corresponding control muscles (Figure 16).

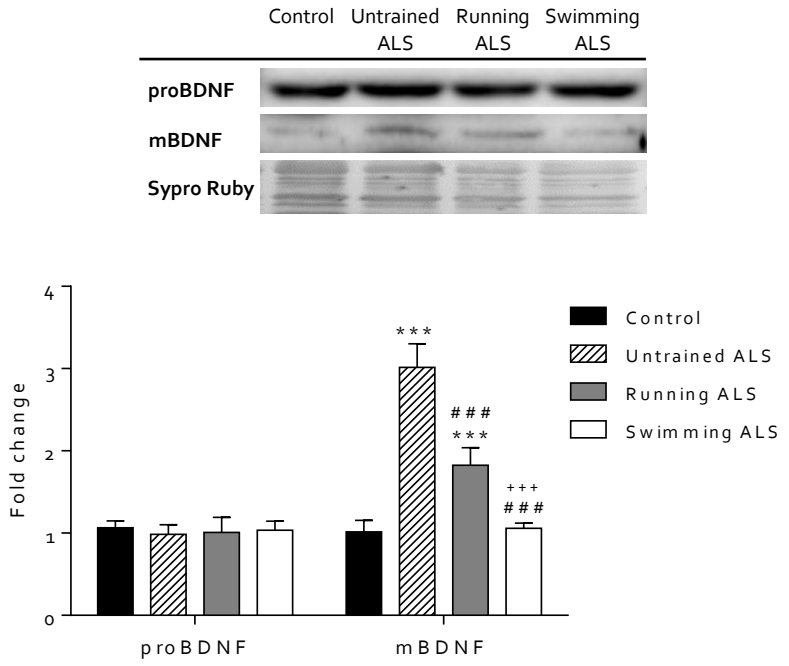


Figure 16. Swimming training efficiently prevented the ALS-induced increase of mBDNF levels in the skeletal muscle of ALS mice. Western blot bands and their quantification show that mBDNF protein levels were significantly increased in ALS mice without affecting proBDNF levels. Both exercises significantly reversed the ALS-induced increase of mBDNF levels but only the swimming-based training efficiently preserved the mBDNF levels in ALS mice close to the corresponding control. Data are represented as means ± SD. *Control versus ALS mice, # Untrained ALS versus trained ALS groups or + Swimming ALS versus Running ALS.

2. Physical exercise prevented the dysregulation of BDNF receptors in the skeletal muscle of ALS mice

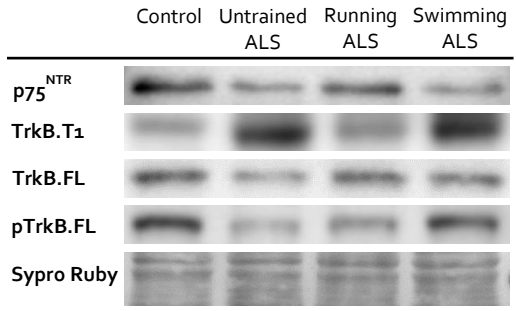
Next, we proceed to analyse how BDNF receptors are modulated in ALS disease. For $p75^{NTR}$ (75kDa), the anti-$p75^{NTR}$ antibody was raised against the amino acids 274–425 of the intracellular domain of the rat protein. Results showed that $p75^{NTR}$ protein levels were significantly decreased (0.53 ± 0.13; $p<0.001$) in skeletal muscle of ALS mice (Figure 17).

To study TrkB.FL and TrkB.T1, we used the anti-TrkB antibody raised against a peptide sequence corresponding to the amino acids 37-75 of TrkB, which belongs to the extracellular domain shared by both TrkB.FL (145-150 kDa) and TrkB.T1 (95-100 kDa). In Chapter I, we confirmed that both receptors are expressed in wild type skeletal muscle, being TrkB-T1 the predominant form. Results showed that TrkB.FL and TrkB.T1 receptors were affected in a different way in skeletal muscle of ALS mice. In particular, we observed that TrkB.T1 levels were significantly increased while TrkB.FL were significantly decreased (2.06 ± 0.08; $p<0.001$ and 0.58 ± 0.19; $p<0.01$; respectively), thus decreasing FL:T1 ratio (Figure 17).

Since TrkB signalling is known to start by phosphorylation of the TrkB.FL (Middlemas et al., 1994) we next determined whether TrkB.FL phosphorylation levels were altered in ALS mice. Specific TrkB.FL phosphorylation was detected with the antibody that specifically recognizes the phosphorylation in the Tyr816 of TrkB.FL (145 kDa), which is known to trigger the PLCγ signalling pathway and sequentially activates PKC (Eide et al., 1996). Phosphorylated TrkB.FL (pTrkB.FL) levels were found to be decreased (0.52 ± 0.13; $p<0.01$) compared to control mice (Figure 17). However, the ratio of phosphorylated and total protein (pTrkB.FL/TrkB.FL) was not affected due to TrkB.FL decrease in a similar proportion.

We next analysed how these receptors were affected in trained ALS mice (Figure 17). We found that running exercise efficiently preserved $p75^{NTR}$ levels close to the control muscles (1.09 ± 0.12; $p>0.05$). However, after swimming protocol, $p75^{NTR}$

levels were still decreased compared to the control similar to untrained ALS mice (0.68 ± 0.02; p<0.01).



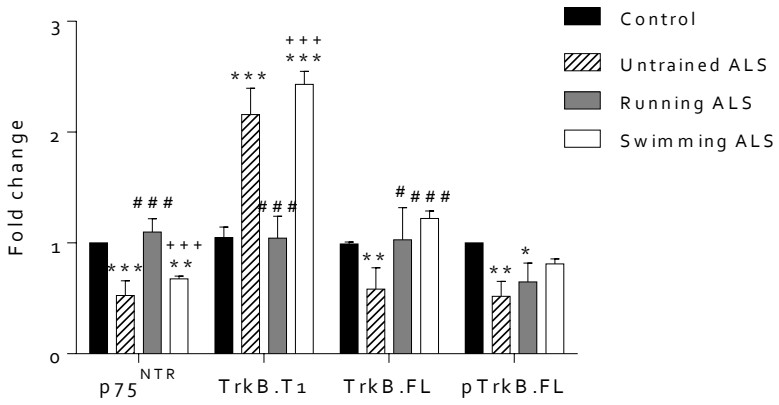


Figure 17. Physical exercise prevented the dysregulation of BDNF receptors in the skeletal muscle of ALS mice. $P75^{NTR}$ and TrkB isoforms were affected in a different way in ALS mice and after exercise. $P75^{NTR}$ and TrkB.FL, as well as pTrkB.FL were decreased in ALS mice. On the contrary, TrkB.T1 was enhanced in ALS mice. Running-based training preserve $p75^{NTR}$ and TrkB.T1 close to the control levels while swimming-based training preserve pTrkB.FL levels. Both training protocols efficiently prevent the downregulation of TrkB.FL protein levels. Data are represented as means ± SD. *Control versus ALS mice, # Untrained ALS versus trained ALS groups or + Swimming ALS versus Running ALS.

Regarding TrkB isoforms, results showed that only the running-based training prevented the ALS-induced increase of TrkB.T1 levels (1.04 ± 0.19; p>0.05). However, after swimming protocol TrkB.T1 levels were still increased compared to the control similar to untrained ALS mice (2.43 ± 0.12; p<0.001). Therefore, ALS

disease increased TrkB.T1 levels and, only the running-based training efficiently preserved the TrkB.T1 levels in ALS mice close to the corresponding control muscles (Figure 17). On the other hand, TrkB.FL protein levels were significantly increased in trained ALS mice compared to ALS mice. Thus, both protocols of exercise significantly prevented the ALS-induced significant decrease of TrkB.FL (running: 1.03 ± 0.29; $p>0.05$; swimming: 1.22 ± 0.07; $p>0.05$) (Figure 17). However, only the running-based training was able to achieve a ratio FL:T1 similar to the control.

Finally, after analysing TrkB phosphorylation (Figure 17), we have found that only the swimming-based training was able to preserve pTrkB levels close to the control (0.82 ± 0.04; $p>0.05$). The running-based training didn't prevent the decrease of its levels since pTrkB was still significantly decreased compared to control muscles (0.64 ± 0.17; $p<0.05$).

Altogether, these results demonstrate that physical exercise prevents the effect of ALS disease over the BDNF signalling pathway in a different way depending on the nature of the exercise. $P75^{NTR}$ and TrkB.FL, as well as pTrkB.FL levels are decreased in favour of increased TrkB.T1 levels in ALS mice. Running-based training is able to preserve p75NTR and TrkB.T1 close to the control levels and swimming-based training preserves pTrkB.FL levels. Both training protocols efficiently prevent the downregulation of TrkB.FL protein levels that occurs in ALS mice.

In conclusion, ALS disease that causes a reduced neuromuscular activity has the BDNF signalling pathway impaired. Physical exercise is able to prevent these modifications and could have a key role in preventing and ameliorating neuromuscular disorders.

DISCUSSION

Enhancement of BDNF signalling has a great potential in therapy for neurological disorders. BDNF not only reduces cell death but also promotes neuronal plasticity, survival and function. However, intrathecally administered BDNF in patients of ALS did not show significant effects on motor function and survival (Ochs et al., 2000) or autonomic nervous system function (Beck et al., 2005). The point is that most neurological disorders not only show a dysregulation of BDNF but also an impairment of its receptors. Thus, an important challenge to this approach is the persistence of aberrant neurotrophic signalling due to a defective function of the TrkB, $p75^{NTR}$ or downstream effectors. Here we demonstrate that BDNF signalling is impaired in ALS skeletal muscle and physical exercise prevents the effect of ALS disease over the BDNF signalling pathway in a different way depending on the nature of the exercise.

BDNF signalling pathway is affected in the skeletal muscle of ALS mice

In ALS patients, BDNF mRNA and protein levels are dramatically upregulated in muscle (Küst et al., 2002) and total TrkB mRNA is increased in the spinal cord, yet, phosphorylation of the TrkB receptor is reduced (Mutoh et al., 2000). Also, a latest study has found that truncated TrkB.T1 deletion significantly slows the onset of motoneuron degeneration in a mouse model of ALS (Yanpallewar et al., 2012). Moreover, $p75^{NTR}$ appears to be involved in ALS pathophysiology (Dupuis et al., 2008; Lowry et al., 2001). When $p75^{NTR}$ is not bound to Trk receptors, it might induce apoptosis in both animal models of ALS and patients. Turner et al. (2004) showed that an antagonist for $p75^{NTR}$ slows progression of ALS in SOD1 animals. Altogether, this suggests that BDNF signalling impairments in ALS are not caused by insufficient NT supply but rather by a mechanism affecting the TrkB response to BDNF. In concordance with that, here we demonstrate an increase of BDNF in the plantaris skeletal muscle from the hind limb of ALS mice. Regarding its receptors, our results show that $p75^{NTR}$ levels are decreased, suggesting a reduced apoptotic

signalling but also reducing the possibility to form heterodimers with TrkB to enhance survival pathways. In addition, both TrkB.FL and its phosphorylation are also decreased in skeletal muscle in favour of increased TrkB.T1 levels. Thus, there is a dysregulation of TrkB isoforms leading to an imbalance between TrkB.T1 and TrkB.FL levels that may be a primary cause or a consequence of impaired neuromuscular function and motoneuron loss. Because of that, it can be suggested that the increase of BDNF in ALS mice could be a compensatory mechanism that is not sufficient to promote neuronal survival of injured motoneurons because of the lack of TrkB.FL available, as it has been already proposed in other studies (Yanpallewar et al., 2012).

One important issue to consider is whether these alterations in BDNF signalling are a cause or a consequence of the disease. It has been shown that in ALS mice there is a pattern of preferential loss of larger MNs innervating faster muscle fibres. It is described that in the fast-twich muscles plantaris and tibialis, only the fast IIB fibres were atrophied in ALS mice. In addition, these muscles suffered a significant fast-to slow transition (Deforges et al., 2009). However, it is not well determined the pattern of expression of BDNF and its receptors depending on the muscle fibre type. So far, Funakoshi et al. (1995) reported greater NT-4 mRNA expression in the predominantly slow soleus muscle compared with the mixed gastrocnemius muscle and localized NT-4 immunoreactivity to slow muscle fibres in adult rats. Gonzalez and Collins (1997) observed a similar differential distribution of BDNF mRNA expression in rat triceps surae muscle (J. E. Dixon, D. McKinnon, M. Gonzalez, and W. F. Collins, unpublished observations). These observations suggest that muscle-derived BDNF and NT-4 are expressed predominantly by slow muscle fibre. Although these conclusions should be confirmed, concerning our results this may indicate that the remaining slow muscle fibres in ALS mice increase the BDNF levels by a compensatory mechanism due to the dysregulation of the BDNF receptors. Also, it may indicate that the fast-to slow transition of muscles fibres that occurs in ALS disease could be related with a transition in the pattern of expression leading to the new converted slow fibres to express BDNF. Further

experiments in our laboratory will be focused to study in detail how BDNF, $p75^{NTR}$ and TrkB isoforms are expressed in fast and slow muscle fibres. That, could help to elucidate the link between the preferential loss of larger MNs and the impaired BDNF signalling in ALS disease to improve targeted therapies

Different training protocols preserve BDNF signalling pathway in the skeletal muscle of ALS mice

In the last few years, there has been increasing evidence of the benefits of physical activity/exercise on the health of the nervous system (van Praag et al., 1999). Increased physical activity has been shown to improve neuromuscular function and alter the structure of the NMJ. Exercise increases the effectivity of the NMJ (Andonian and Fahim, 1987; Tomas et al., 1993, 1997), the total area of both pre- and postsynaptic elements (Deschenes et al., 1993), and the amount of ACh released (Dorlöchter et al., 1991). Thus, as another therapeutic approach, training exercise has been proposed to provide benefits during the early or late stages of ALS. The potential positive effect of physical activity on ALS has been tested in mouse models of ALS but conflicting results have clouded the role of physical exercise. Regular moderate intensity exercise has been reported to have neuroprotective effects delaying the onset of the disease and/or its progression with a modest increase in the survival of transgenic mice (Kaspar et al., 2005; Kirkinezos et al., 2003; Veldink et al., 2003). When regular moderate intensity exercise was combined with insulin-like growth factor (IGF-1) treatment, a strong synergistic effect was reported (Kaspar et al., 2005). Lifetime exposure of transgenic ALS mice to vigorous physical activity based on a 10h/day on a motor-driven running wheel showed that exercise did not promote hasten the progression of motor neuron degeneration (Liebetanz et al., 2004) however, in the same transgenic ALS mice (SOD1G93A), high intensity endurance treadmill exercise for 45 min/day, 5 times/week progressive increased from 9 to 22 m/min hastened the onset of weakness and death (Mahoney et al., 2004). Given these contradictory results, it is important to address how different levels of exercise could affect to

motor neuron viability. In addition, several clinical studies in ALS patients have demonstrated the value of moderate exercise in ameliorating disease symptoms and improving functionality (Bello-Haas et al., 2007; Drory et al., 2001; Pinto et al., 1999).

Although the molecular mechanism(s) underlying the exercise-induced effects is still unknown, it could be a link between the motoneuron activation, the adaptation of neuron intrinsic properties and neuroprotection. Deforges et al. (2009) presented the differential effect of a running based and a swimming based exercise protocols in ALS mice due to the activation of different sub-population of MN. Hence, here we analyse these two types of exercise: (1) swimming-based training, which can be described as a high movement amplitude and frequency exercise that preferentially activates large MNs, belonging to fast motor units. Moreover, in plantaris muscle of ALS mice, swimming protocol efficiently prevents the fast-to-slow transition of fibres types that occurs during the disease, preserving the ALS muscle phenotype close to the controls muscles. (2) running-based training, which can be described as a low movement amplitude and frequency exercise that preferentially activates small MNs belonging to slow motor units. However, this protocol is associated with a fast-to-slow transition of fibres types in plantaris muscle, worsening the effects of the disease in ALS mice (Deforges et al., 2009).
Here we show that both training protocols significantly reversed the ALS-induced increase of mBDNF levels but only the swimming-based training efficiently preserved the mBDNF levels in ALS mice close to the corresponding control muscles. As described above, BDNF seems to be preferentially expressed in slow muscle fibres. The fact that swimming protocol preserve mBDNF close to the control could be link to the prevention of the fast-to-slow transition of muscles fibres that occurs with this training in ALS disease. Swimming protocol preventing the transition, would stop that a new converted slow muscle fibres population starts to express BDNF, thus balancing BDNF expression.
On the contrary, only the running-based training efficiently preserved the $p75^{NTR}$ and TrkB.T1 levels in ALS mice close to the corresponding control muscles.

Regarding TrkB.FL and its phosphorylation, both training protocols significantly prevent the ALS-induced decrease of TrkB.FL and pTrkB levels, but only the swimming-based training preserve pTrkB levels close to the control. It is interesting that running protocol impacts more efficiently over "negative" receptors as $p75^{NTR}$ and TrkB.T1 but swimming protocols is more efficient to regulate "positive" receptors, TrkB.FL and its phosphorylation. This could be associated with the different MN and muscle fibre survival in each exercise and/or the ratio of expression of these receptors depending on the fibre type. However, further experiments will be done in our laboratory to study in detail how BDNF, $p75^{NTR}$ and TrkB isoforms are expressed in fast and slow muscle fibres.

The results of this Chapter II are summarized in Figure 18. Therefore, here we demonstrate the impairment of BDNF signalling pathway in skeletal muscle of ALS disease. These alterations could be prevented by physical exercise but in a different way depending on the intensity and nature of exercise. Thus, there is a needed to further define what type, intensity, and duration of exercise may prove truly beneficial in ALS.

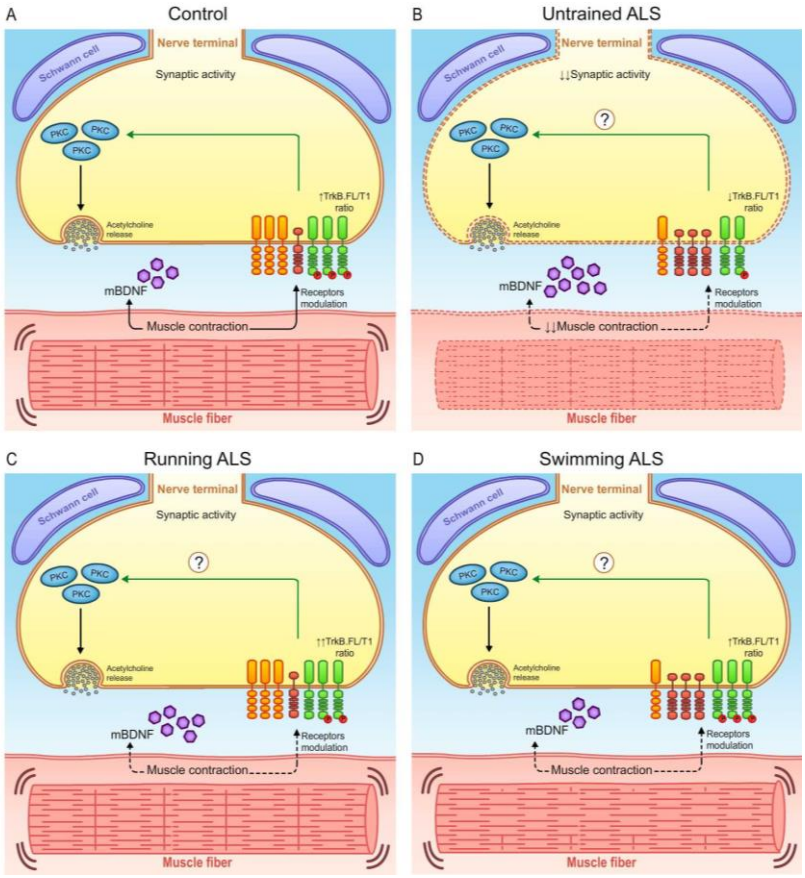


Figure 18. Graphical overview of BDNF signalling in ALS disease. **(A)** BDNF signalling at the neuromuscular junction in Control mice; **(B)** Untrained ALS mice; **(C)** Running mice and **(D)** Swimming mice. **(B)** mBDNF levels are increased in ALS disease and its receptors are dysregulated. $P75^{NTR}$ (in orange), TrkB.FL (in green) and its phosphorylation are decreased while TrkB.T1 levels are increased (in red) in ALS disease. **(C)** After running training, mBDNF levels decreased compared to the ALS situation but they were still increased compared to the control. Regarding its receptors, the reduction of $P75^{NTR}$ and TrkB.FL levels that happened in ALS, was prevented after running training, keeping the levels close to the control. Similarly, running training prevented the ALS-induced increase of TrkB.T1 levels. However, TrkB phosphorylation was still significantly decreased compared to the control after running protocol. **(D)** The swimming-based training was the only that efficiently preserved mBDNF and TrkB.FL phosphorylation levels close to the control, in addition to preserve TrkB.FL total protein levels. However, after swimming training, $P75^{NTR}$ and TrkB.T1 levels were still altered by the disease compared to the control mice.

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E. GENERAL DISCUSSION

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GENERAL DISCUSSION

The neuromuscular system is a complex and interconnected network that links the nervous system with muscles located throughout the body. It is important to address how it is modulated to increase the knowledge about some diseases, such as ALS, in which the progressive interruption, at the neuromuscular junction level, of the connection between nerve and muscle leads to the pathological non-communication of the two tissues.

Skeletal muscle differentiation and function is controlled by a variety of signals from different sources. Innervating MNs and contacting Schwann cells "tell" skeletal muscle cells to grow, to differentiate and how they should function, as a postsynaptic component. Conversely, skeletal muscle provides signals, including neurotrophins (NTs), that regulates in the presynaptic component the survival and function of MNs during development, maintenance and/or injury (Huang and Reichardt, 2001). It is well known that NTs as BDNF, are widely regulated by neuromuscular activity and binding to its receptors activates many different pathways that could impact on NMJ functionality, for example activating presynaptic PKCs signalling. **But which could be the mechanisms that coordinates pre- and postsynaptic activities to modulate neuromuscular function through regulation of BDNF and PKC signalling?**

In the last few years, there has been increasing evidence of the benefits of physical activity/exercise on the health of the nervous system (van Praag et al., 1999). Increased physical activity has been shown to improve neuromuscular function and alter the structure of the NMJ. Exercise increases the effectivity of the NMJ (Andonian and Fahim, 1987; Tomas et al., 1993, 1997), the total area of both pre- and postsynaptic elements (Deschenes et al., 1993), and the amount of ACh released (Dorlöchter et al., 1991). These effects could be conducted by NTs since increased exercise training has also been shown to have effects on NTs expression in mammalian skeletal muscle (Sakuma and Yamaguchi, 2011). Specifically, it is well-accepted the preponderance of BDNF in mediating these benefits. In the

neuromuscular system, however, whether skeletal muscle *in vivo* increases its production and/or release of BDNF by synaptic activity, muscle contraction or some combination of the two, remained unclear.

Muscle contraction enhances the activity-dependent increase of mBDNF and downregulates TrKB.T1 levels in skeletal muscle

BDNF is strongly expressed in the brain (Huang and Reichardt, 2003) and to a lesser extent in skeletal muscle (Matthews et al., 2009) and it plays a key role in regulating survival, growth and maintenance of neurons (Snider, 1998). It is initially synthesized as a precursor protein (proBDNF) and subsequently cleaved into mBDNF.

It is established that BDNF transcription, translation and secretion is strongly regulated by neural activity. Specifically, exercise training increases BDNF expression in spinal cord and skeletal muscle in rodents (Cuppini et al., 2007; Gómez-Pinilla et al., 2001, 2002, 2012; Zoladz and Pilc, 2010) and basal neuromuscular activity is required to maintain normal levels of BDNF in the NMJ (Gómez-Pinilla et al., 2002). **But does synaptic activity and/or muscle contraction per se modulate BDNF mRNA and protein levels in skeletal muscle?** Liem et al. (2001) showed that mRNA is only located inside myocytes and Matthews et al. (2009) demonstrated that BDNF mRNA and protein expression is increased in muscle cells that are electrically stimulated. In this thesis, we have demonstrated that BDNF mRNA expression (IV, VI, VIII and IX activity dependent exons) is not affected after 30 minutes of synaptic activity or muscle contraction. However, both synaptic activity and muscle contraction increase mBDNF protein levels without alter proBDNF. Specifically, muscle contraction *per se* is able to increase mBDNF levels over and above what nerve transmission alone can enhance. This, suggests a postsynaptic origin through BDNF mRNA translation that is directly linked to myofibril contraction. Other evidences showed that following exercise, BDNF mRNA expression is consistently increased in the CNS and PNS in a dose-dependent manner (Adlard et al., 2004; Gómez-Pinilla et al., 2002). In Sprague-

Dawley rats exercised on a treadmill, BDNF mRNA expression is enhanced in soleus muscle following 5 days but not 10 days of exercise. NT-4/5 and TrkB mRNA levels are not affected at either time point (Gómez-Pinilla et al., 2001). Other study of the same authors, after voluntary exercise, BDNF mRNA expression and protein levels are enhanced in soleus muscle of rats following 3 and 7 days of exercise (Gómez-Pinilla et al., 2002). Our results suggest that the stimulation protocol used is not sufficient to increase BDNF mRNA in muscle. Thus, here we show that short-term acute neuromuscular activity enhances muscle BDNF protein levels by promoting its translation and/or maturation (e.g., through increased protease activity) allowing a build-up of mBDNF and leaving net pro-BDNF levels unchanged.

BDNF and NT-4 can initiate intracellular signalling through the same cell surface receptor, TrkB (Klein et al., 1991, 1992). NT-4 expression is also dependent on the activity of neuromuscular synapses. Electrical stimulation of motor nerves enhances NT-4/5 expression in skeletal muscle, and a blockade of neuromuscular endplates with bungarotoxin lead to reduced NT-4/5 expression (Funakoshi et al., 1995). However, it is of interest that NT-4/5 has not been evaluated accurately following similar exercise. One study has addressed the issue of exercise-induced NT-4/5 expression, finding no significant elevation of NT-4/5 protein levels in the vastus lateralis of two aerobically trained individuals. In concordance with that, our results show that after 30 minutes of electrical stimulation with and without resulting muscle contraction, NT4 protein levels are not affected.

BDNF isoforms, pro-BDNF and mBDNF, bind distinct receptors to mediate divergent neuronal actions (Hempstead, 2006; Je et al., 2013; Lu, 2003; Yang et al., 2009). Pro-BDNF interacts preferentially with $p75^{NTR}$, whereas mBDNF selectively binds and activates its specific receptor TrkB. Having demonstrated that BDNF expression is modulated by synaptic activity and muscle contraction *per se,* it brings us to the next question: **Are BDNF receptors modulated by activity?** TrkB has been implicated in the maintenance of the synaptic function and the structural integrity in adult muscular synapses (Mantilla et al., 2014). There are several alternatively spliced isoforms of TrkB with the same affinity to NTs, including

TrkB.FL and two truncated TrkB isoforms T1 and T2 (TrkB.T1 and TrkB.T2), which lack part of the intracellular kinase domain (Middlemas et al., 1991; Reichardt, 2006). Evidence suggests that heterodimers of TrkB.FL with the truncated isoforms inhibit trans-autophosphorylation of TrkB.FL, reducing BDNF signalling (Baxter et al., 1997; Dorsey et al., 2012; Eide et al., 1996; Rose et al., 2003; Wong and Garner, 2012). TrkB.T2 is a variant mainly predominant in the brain tissue and does not appear to have individual signalling ability (Stoilov et al., 2002). TrkB.T1 is the main truncated isoform in the skeletal muscle and some studies suggest unique signalling roles for TrkB.T1, for example, by modulating Ca^{2+} signalling mechanisms in astrocytes (Rose et al., 2003). Other evidence suggests that TrkB.T1 acts in a dominant negative fashion to decrease signalling through TrkB.FL (Eide et al., 1996; Gonzalez et al., 1999; Haapasalo et al., 2001). Dorsey et al, (2012) demonstrated that the deletion of TrkB.T1 increases neurotrophin-dependent activation of downstream signalling targets (e.g., Akt and p70/sk6) and increases contractibility.

In addition, physical activity has been reported to increase TrkB.FL mRNA levels in skeletal muscles and spinal cord (Gómez-Pinilla et al., 2002; Skup et al., 2002; Zoladz and Pilc, 2010). In this thesis, we demonstrate in concordance with previous results, that there is a predominance of TrkB.T1 levels over TrkB.FL in skeletal muscle. Moreover, as a novel finding we found that muscle contraction *per se* downregulates TrkB.T1 (decreasing the ratio to FL:T1) without changing TrkB.FL or $p75^{NTR}$ levels. Therefore, it appears that the ratio between TrkB.FL and TrkB.T1 could determine the net effect of BDNF signalling at the neuromuscular system.

BDNF binds to the TrkB.FL and activate the intrinsic tyrosine kinase domain, leading to autophosphorylation in the activation loop (tyr701, tyr706 and tyr707; Guiton et al., 1994; Huang and Reichardt, 2003). The phosphorylation of these residues can lead to the transphosphorylation of others tyrosine residues (Cunningham et al., 1997; Friedman and Greene, 1999) being tyr515 and tyr816 the most extensively studied phosphorylation sites (Middlemas et al., 1994; Segal et al., 1996). Neuronal activity has been shown to rapidly activate TrkB and

potentiate its signalling, an effect attributed to activity dependent secretion of BDNF (Meyer-Franke et al., 1998; Aloyz et al., 1999; Patterson et al., 2001). Here we show that synaptic activity induces a quick increase in pTrkB.FL after 1–10 min but returned to baseline by 30 min. However, muscle contraction decreases phosphorylation of TrkB.FL after 1–10 min by increasing phosphatase activity, but returned to baseline after 30 min. These results suggest a complex mechanism regulating phosphorylation of TrkB.FL and it needs further investigation in the future.

Altogether, our results indicate that mBDNF levels are enhanced by synaptic activity and muscle contraction *per se*, the latter further enhancing the synaptic activity-dependent increase of mBDNF. It is a novel finding that synaptic activity enhances BDNF levels that may act as a paracrine or endocrine signal. Moreover, it seems that when muscle contraction is present, BDNF signalling output is, to a great extent, caused by a mechanism affecting the TrkB ratio (FL:T1) rather than BDNF expression. Here we also demonstrate that the modulation of BDNF signalling by neuromuscular activity is determined by its receptor in a time-dependent at the NMJ. At short times (1–10 min), synaptic activity and muscle contraction regulate phosphorylation of TrkB.FL (tyr816) and, at longer times (30 min), the regulation is mediated by an effect on TrkB.T1.

BDNF/TrkB signalling pathway is impaired in the skeletal muscle of ALS mice and physical exercise can prevent it

We have concluded that BDNF/TrkB signalling is highly regulated by synaptic activity and muscle contraction in the NMJ. Thus, **could BDNF/TrkB signalling represent a new opportunity for intervention across neuromuscular diseases that are characterized by deficits in presynaptic activity and muscle contractility such as ALS?** Therefore, first, it is important to address how is modulated the activity-dependent BDNF/TrkB signalling pathway in ALS disease, in which muscle contraction is decreased. ALS is a progressive neurodegenerative disorder characterized by the selective degeneration of motoneurons and NMJ

which causes muscular weakness, atrophy and spasticity among others symptoms (Kiernan et al., 2011; National Institute of Neurological Disorders and Stroke, 2017; Moloney et al., 2014). Despite substantial number of approaches have been tested clinically, therapeutic options are limited. As neurotrophic factors are known to promote survival of neurons, they have been investigated pre-clinically and clinically for the treatment of ALS, without success.

Different evidence has shown that BDNF has strong pro-survival effects with promising results on rescuing axotomized motoneurons (Giménez y Ribotta et al., 1997; Sendtner et al., 1992; Yan et al., 1992) and on improving the phenotype of wobbler mutant mice (an ALS mice model; Ikeda et al., 1995). In addition, BDNF protects neurons from *in vivo* excitotoxicity (Bemelmans et al., 2006), a mode of action of relevance to ALS (Henriques et al., 2010). However, in ALS patients, BDNF is significantly increased in skeletal muscle and spinal cord (Küst et al., 2002). One of the reasons that could explain the failure of BDNF in clinical trials is due to its binding to $p75^{NTR}$, that it appears to be involved in ALS pathophysiology (Dupuis et al., 2008; Lowry et al., 2001). It is known that $p75^{NTR}$ can induce proapoptotic signals but can also form heterodimers with Trk receptors to enhance neurotrophin response, thus increasing survival signalling (Bibel et al., 1999; Chao and Hempstead, 1995; Davies et al., 1993). Thus, when $p75^{NTR}$ is not bound to Trk receptors, it might induce apoptosis in both animal models of ALS and patients. Turner et al. (2004) showed that an antagonist for $p75^{NTR}$ slows progression of ALS in SOD1 animals. Moreover, experiments in mutant SOD1G93A mice where TrkB-T1 is deleted showed that the onset of the disease is delayed and the motoneuron loss is slowed down. In addition, these mice perform better in mobility tests at the end stage of the disease in relation with normal mutant SOD1G93A mice (Yanpallewar et al., 2012). Also, in ALS patients TrkB.FL phosphorylation is dramatically decreased in the spinal cord (Küst et al., 2002; Mutoh et al., 2000).

In this thesis, we confirm the increase of BDNF in the plantaris skeletal muscle from the hind limb of ALS mice. Surprisingly, our results show that $p75^{NTR}$ levels are

decreased, suggesting a reduced apoptotic signalling but also a reduced chance to binding to TrkB to enhance survival pathways. Moreover, we demonstrate that phosphorylated TrkB.FL is also decreased in skeletal muscle (due to a loss of total TrkB.FL protein levels). In addition, TrkB-T1 is strongly increased in ALS mice, which suggests that a dysregulation of the balance between TrkB.T1 and TrkB.FL levels may lead to an impaired neuromuscular function and motoneuron loss. In concordance with our previous results discussed above in which muscle contraction decreases TrkB.T1 levels, in a context where muscle contraction is reduced by the disease, it is not able to reduce TrkB.T1 levels. It seems that TrkB signalling impairments in ALS are not caused by insufficient NT supply but rather by a mechanism affecting the TrkB response to BDNF, similar to our previous results (see above). Therefore, over-presence of TrkB.T1 may limits the pro-survival effects of BDNF, decreasing TrkB.FL phosphorylation and activation causing a direct impact on the signal transduction. Because of that, it can be suggested that the increase of BDNF in ALS model mice could be a compensatory mechanism that is not sufficient to promote neuronal survival of injured motoneurons because of the lack of TrkB.FL available, as it has been already proposed in other studies (Yanpallewar et al., 2012).

It is interesting to note that studies in ALS mice has shown that there is a pattern in ALS disease of preferential loss of larger MNs innervating faster muscle fibres. Thus, MNs innervating the slower muscle fibres are more resistant in the SOD1G93A mouse model of ALS than those innervating the faster ones (Hegedus et al., 2007, 2008). It is described that in the fast-twich muscles plantaris and tibialis, only the fast IIB fibres were atrophied in ALS mice. In addition, these muscles suffered a significant fast-to slow transition from fast-twitch type II fibres to slow-twitch type I fibres and, within the type II fibre population, from type IIB/IIX to IIA fibres (Deforges et al., 2009). Importantly, our studies have been performed in plantaris muscle, a good model to attempt a first approach at studying skeletal muscle degeneration in ALS. So far, Funakoshi et al. (1995) reported greater NT-4 mRNA expression in the predominantly slow soleus muscle compared with the

mixed gastrocnemius muscle and localized NT-4 immunoreactivity to slow muscle fibres in adult rats. Gonzalez and Collins (1997) observed a similar differential distribution of BDNF mRNA expression in rat triceps surae muscle (J. E. Dixon, D. McKinnon, M. Gonzalez, and W. F. Collins, unpublished observations). These observations suggest that muscle-derived BDNF and NT-4 are expressed predominantly by slow muscle fibre. Although these conclusions should be confirmed, concerning our results this may indicate that the remaining slow muscle fibres in ALS mice increase the BDNF levels by a compensatory mechanism due to the dysregulation of the BDNF receptors. Also, it may indicate that the fast-to slow transition of muscles fibres that occurs in ALS disease could be related with a transition in the pattern of expression leading to the new converted slow fibres to express BDNF. Further experiments in our laboratory will be focused to study in detail how BDNF, $p75^{NTR}$ and TrkB isoforms are expressed in fast and slow muscle fibres. That, could help to elucidate the link between the preferential loss of larger MNs and the impaired BDNF signalling in ALS disease to improve targeted therapies.

As another therapeutic approach, training exercise has been proposed to provide benefits during the early or late stages of ALS (Drory et al., 2001; Pinto et al., 1999). It promotes cellular adaptations in the brain, spinal cord, and skeletal muscles that could counteract the oxidative stress complication in ALS (Husain and Somani, 1997). In skeletal muscle, training reduces oxidative stress following exercise (Miyazaki et al., 2001), increases the mitochondrial capacity (Holloszy et al., 1970) and increases the expression of neurotrophic factors (Gómez-Pinilla et al., 2001) that could prevent motoneuron degeneration, preserve muscle innervation and inhibit muscle atrophy (Acsadi et al., 2002; Manabe et al., 2002; Sun et al., 2002). Several clinical studies in ALS patients have demonstrated the value of moderate exercise in ameliorating disease symptoms and improving functionality (Bello-Haas et al., 2007; Drory et al., 2001; Pinto et al., 1999).

Although the molecular mechanism(s) underlying the exercise-induced effects is still unknown, it could be a link between the motoneuron activation, the adaptation

of neuron intrinsic properties and neuroprotection. Thus, **how could be modulated BDNF signalling by exercise in ALS mice? It could prevent the BDNF/TrkB signalling impairment that occurs in ALS skeletal muscle? Could different type of exercise with different intensity differentially impact over BDNF signalling in ALS mice?**

Deforges et al. (2009) reported for the first time, that exercise impacts on all neural cell distribution in ALS spinal cord with an efficiency dependent on the nature of the exercise. Moreover, different types of exercise can be related with specific MN and muscle fibre types survival. In this thesis, we have analysed two types of exercise: (1) swimming-based training, which can be described as a high movement amplitude and frequency exercise that preferentially activates large MNs, belonging to fast motor units and (2) running-based training, which can be described as a low movement amplitude and frequency exercise that preferentially activates small MNs belonging to slow motor units (Gronard et al., 2008). Taken together, in the same way that neurons and muscle fibres are selectively activated by physical exercise, they are also differentially protected against cell death. In plantaris muscle of ALS mice, swimming protocol efficiently prevents the fast-to-slow transition of fibres types that occurs during the disease, preserving the ALS muscle phenotype close to the controls muscles (Deforges et al., 2009). However, running protocol is associated with a fast-to-slow transition of fibres types in plantaris muscle, worsening the effects of the disease in ALS mice (Deforges et al., 2009).

Here we show that both training protocols significantly reversed the ALS-induced increase of mBDNF levels but only the swimming-based training efficiently preserved the mBDNF levels in ALS mice close to the corresponding control muscles. However, **are the swimming training effects, preserving BDNF expression, only a consequence related with the protection of fast fibres and large MN? Could be these molecular changes the primary cause of this pattern of protection?** As described above, BDNF seems to be preferentially expressed in slow muscle fibres. The fact that swimming protocol preserve mBDNF close to the

control could be link to the prevention of the fast-to-slow transition of muscles fibres that occurs with this training in ALS disease. Swimming protocol preventing the transition, would stop that a new converted slow muscle fibres population starts to express BDNF, thus balancing BDNF expression.

On the contrary, only the running-based training efficiently preserved the $p75^{NTR}$ and TrkB.T1 levels in ALS mice close to the corresponding control muscles. Regarding TrkB.FL and its phosphorylation, both training protocols significantly prevent the ALS-induced decrease of TrkB.FL and pTrkB levels, but only the swimming-based training preserve pTrkB levels close to the control. It is interesting that running protocol impacts more efficiently over "negative" receptors as $p75^{NTR}$ and TrkB.T1 but swimming protocols is more efficient to regulate "positive" receptors, TrkB.FL and its phosphorylation. This could be also associated with the different MN and muscle fibre survival in each exercise and/or the ratio of expression of these receptors depending on the fibre type. Thus, as stated above, it is necessary to know with more detail how BDNF, $p75^{NTR}$ and TrkB are differentially express in slow and fast muscles and MNs. This may help to elucidate if these changes in TrkB/BDNF expression in ALS mice are due to the preferentially loss of fast fibres and large MN or if these molecular changes could be the primary cause of this lost. So far, here we demonstrate an altered expression pattern of mBDNF and its receptors in ALS disease that could be prevented with physical exercise. Thus, BDNF and mainly its receptors, represent a new opportunity for intervention across neuromuscular diseases that are characterized by deficits in presynaptic activity and muscle contractility such as ALS.

Muscle contraction enhances cPKC isoforms (α, β) through BDNF/TrkB pathway

One of the most important signalling pathways that triggers BDNF binding to TrkB, is PLCy, which produces DAG and IP_3 (Carpenter and Ji, 1999). IP_3 leads to release of Ca^{2+}, as a result, mature PKCs are tethered to the membrane leading to a massive conformational change, allowing for substrate binding, phosphorylation and the activation of downstream signalling effectors (Colón-González and

Kazanietz, 2006; Griner and Kazanietz, 2007). PKC isoforms are different regulated and localized in the NMJ. nPKCε and cPKCβI are located exclusively in the nerve terminal and PKCα is present in the three components of the NMJ (Besalduch et al., 2010; Obis et al., 2015a). Obis et al. (2015) showed that both synaptic activity and muscle contraction can induce changes in the expression of the presynaptic nPKCε. As previously published (Besalduch et al., 2010), our results show that cPKCα and cPKCβI, are similarly regulated by neuromuscular activity. Synaptic activity decreases cPKCα and cPKCβI by an activation-induced degradation mechanism and muscle contraction increases its protein levels. The latter, could be associated with an increased cPKCα and cPKCβI protein synthesis or a decreased protein degradation. As a new finding, in this thesis we demonstrate that also PKC phosphorylation is regulated by synaptic activity. Synaptic activity directly increases cPKCα and cPKCβI phosphorylation probably linked to the regulation of neurotransmission process. However, muscle contraction further increases cPKCα and cPKCβI phosphorylation due to the increase of their synthesis.

That muscle contraction impact over proteins that are exclusively located in the nerve terminal (nPKCε and cPKCβI) suggest the existence of a retrograde factor influenced by neuromuscular activity that could modulate presynaptic PKC at the NMJ. **Could be BDNF this retrograde factor that binding to TrkB regulate cPKCα and cPKCβI and its phosphorylation at the NMJ?** Several studies demonstrated that BDNF-induced potentiation of presynaptic vesicle release requires TrkB phosphorylation and PLC activation (Kleiman et al., 2000), in turn activating PKC. It has been suggested that neuromuscular activity increases retrograde transport of BDNF from the muscle to the spinal cord (Curtis et al., 1998; Koliatsos et al., 1993; Sagot et al., 1998; Yan et al., 1992). Moreover, previous studies in our laboratory suggested that TrkB is involved in the activity-dependent modulation of presynaptic nPKCε (Obis et al., 2015a). However, there was lack of knowledge of BDNF/TrkB modulation regarding PKCα and βI. Here we demonstrate that BDNF regulation over these PKCs is directed by both synaptic activity and muscle contraction in opposite directions, suggesting distinct roles of presynaptic vs. postsynaptic induced-BDNF. The apparent distinct roles are consequence of the

muscle contraction-induced decrease of TrkB.T1. Muscle contraction increases TrkB.FL/T1 ratio and the reduction of the T1 dominant negative fashion over FL signalling upregulates presynaptic cPKCs (increasing its synthesis or decreasing the activation-induced degradation). In this way, our data provide new evidence indicating the essential role that synaptic activity-induced muscle contraction plays in the regulation of PKCα and βI isoforms through BDNF/TrkB signalling pathway. However, our results also show that PKC phosphorylation is not directly linked to BDNF/TrkB pathway. This suggests that BDNF/TrkB is involved in PKC activation once they are phosphorylated and mature.

The complex mechanisms involved in cPKC activation have been extensively studied (Newton, 2003, 2010; Parekh et al., 2000) and it is required different steps before is able to phosphorylate its substrates. First, new synthetized PKCs undergoes a process of maturation (Newton, 2010; Parekh et al., 2000) that includes a series of phosphorylation steps in the Aloop, TM and the HM. The first phosphorylation is mediated by PDK1 and occurs at the Aloop site. Then, the TM and the HM are exposed in the C-terminal domain and are autophosphorylated leading to stabilization of the enzyme. The mature PKC is now 'primed' for activation (by DAG and Ca^{2+}) and is released into the cytosol and kept in an inactive conformation (Griner and Kazanietz, 2007; Oancea and Meyer, 1998; Violin et al., 2003). However, recent finding showed that PKC phosphorylation (in Aloop, HM and TM), may occur much later during their life cycle and predominantly in response to cellular stimulation. Growth factor-dependence HM phosphorylation is reported in nPKCε, whereas other reports showed that HM phosphorylation of cPKCα, nPKCδ and nPKCε are rapamycin sensitive (Behn-Krappa and Newton, 1999; Cenni et al., 2002; Parekh et al., 1999; Ziegler et al., 1999). Therefore, PKCs can also exist in non/hypophosphorylated forms, with cellular stimulation resulting in inducible phosphorylation (Freeley et al., 2011; Osto et al., 2008; Wang et al., 2007; Zhou et al., 2003). In this thesis, we demonstrate that phosphorylation of PKCα and βI is not a constitutive mechanism because it can be induced by synaptic activity. Once intracellular calcium and DAG increase, mature PKCs are tethered to

the membrane leading to a massive conformational change that releases the pseudosubstrate domain from the substrate-binding site, allowing for substrate binding, phosphorylation and the activation of downstream signalling effectors (Colón-González and Kazanietz, 2006; Griner and Kazanietz, 2007). Finally, after their activation, PKCs are downregulated through a poorly understood mechanism.

Since PDK1 is the main upstream kinase for PKC that mediates its phosphorylation and maturation, **could be PDK1 also modulated by synaptic activity?** The action of PDK1 on PKC signalling has been extensively studied but how PDK1 activity is regulated in the NMJ remained unknown.

Synaptic activity translocates presynaptic PDK1 and cPKCβI to the plasma membrane of skeletal muscle

PDK1 is a serine and threonine kinase that belongs to the AGC superfamily of protein kinases and it was identified as the upstream kinase of Akt1 and PKC, among others (Balendran et al., 2000; Chou et al., 1998; Downward, 2004; Keranen et al., 1995; Le Good et al., 1998; Sonnenburg et al., 2001). This establishes PDK1 as a central activator of multiple signalling pathways coupled to a large number of growth-promoting stimuli. PDK1 contains a carboxyl-terminal PH domain that binds to the lipid products of PI3K, PI(3,4,5)P_3 and PI(3,4) and to other phosphoinositides such us PI(4,5)P_2 (Alessi et al., 1997a; Currie et al., 1999). Its activation is dependent on its PH domain binding to plasma membrane PI (3,4,5)P_3, where is able to autophosphorylate itself and to phosphorylate its substrates. In PDK1, Ser25, Ser241, Ser393, Ser396 and Ser410 were first shown to be phosphorylated *in vivo*, but their phosphorylation level is not modulated by growth factor stimulation (Alessi et al., 1997b). Therefore, PDK1 seems to be constitutively active, owing to the autophosphorylation ability of the Ser241 residue (Vanhaesebroeck and Alessi, 2000). However, it is still controversial, since other studies suggest that signalling pathways activated by IGF-1 can further increase the degree of PDK1 phosphorylation on these sites (Scheid et al., 2005). Alessi et al.

(1997) as well as Wick et al. (2003) showed that only Ser241 (in the kinase domain) is indispensable for PDK1 activity, and phosphorylation of PDK1 in S241 is catalysed by an autophosphorylation reaction in trans (Wick et al., 2003). Surprisingly, Ser241 phosphorylation is resistant to dephosphorylation by protein phosphatase2A (PP2A), indicating that it was not accessible by phosphatases (Casamayor et al., 1999). Here we show that neuromuscular activity does not affect total PDK1 levels nor its phosphorylation in Ser241 site, in skeletal muscle total lysates. This indicates that there is a stable pool of PDK1 at the NMJ probably related with the constitutive activation of this enzyme. Later, other major autophosphorylation site (Thr51) in the PH domain appeared to be significant for PDK1 phosphorylation and activity, in the presence of PI(3,4,5) P_3 (Gao and Harris, 2006). This implied a strong regulatory role for the PH domain in the activation of PDK1 upon PI(3,4,5)P_3 binding, importantly to take into account.

Different substrates of PDK1 are phosphorylated and activated by distinct regulatory mechanisms. **But how can a constitutive kinase as PDK1, dictate the function of so many different downstream kinases?** In Newton (2003) is described an efficient mechanism: the substrate dictates when it needs to be phosphorylated by PDK1. PDK1 substrates need to be at the right location and in the right conformation before they can be phosphorylated by PDK1, whose intrinsic activity appears to be constitutive. For example, Akt needs to be in the membrane through binding to IP(3,4,5)P_3 via its PH domain, and it is subsequently phosphorylated at its residue Thr308 and activated by PDK1, which itself is also associated to the membrane via PH domain-dependent binding to PI(3,4,5)P_3 (Hemmings and Restuccia, 2012). It is the most significant example of PI(3,4,5)P_3-dependent protein activation. However, for other substrates of PDK1 lacking a PH domain, such as PKCs, the interaction and subsequent activation was shown to be independent of phosphoinositides (Sonnenburg et al., 2001). Rather, PKC becomes a substrate for PDK1 when it is membrane-bound and in an 'open' conformation in which the pseudosubstrate is removed from the substrate-binding cavity, thus unmasking the activation loop site to allow phosphorylation by PDK-1 (Dutil and

Newton, 2000). Therefore, it is important to detail our previous results and to know how cPKCβI is modulated in the membrane fraction. Moreover, **could be PDK1 and cPKCβI regulated in connection by neuromuscular activity in the membrane fraction of skeletal muscle?**

Here we demonstrate that PDK1 is located exclusively in the nerve terminal at the NMJ. It is surprising that while several PKC isoforms are located in the different cells of the rat NMJ (Besalduch et al., 2010, 2013; Lanuza et al., 2014; Obis et al., 2015a; Perkins et al., 2001), PDK1 is located exclusively in the nerve terminal. This fact may be related with a specific role of this protein in priming presynaptic kinases, such cPKCβI. Moreover, our results demonstrate that synaptic activity promotes a significant increase of pPDK1 in the membrane fraction, coincident with a significant increase of pcPKCβI also in the membrane fraction. This might indicate that synaptic activity increases PDK1 function to phosphorylate cPKCβI, priming PKC for its substrate binding, phosphorylation and the activation of downstream signalling effectors. Ikenoue et al. (2008) suggested also an essential physiological function of mTORC2 in the regulation of PKC by promoting phosphorylation and maturation of the kinases. So, it is important to consider the possible role of mTORC in the regulation of pPKCs. The increase of pcPKCβI in the membrane after synaptic activity is accompanied by a significant decrease of the total cPKCβI indicating that mature PKC is downregulated after its activation. This is in concordance with our previous results in skeletal muscle total lysate, detailing that is in the membrane where the downregulation occurs. Furthermore, our previous results described above showed that total but not phosphorylated cPKCβI protein levels depends on synaptic activity-induced BDNF/TrkB signalling at the NMJ indicating that PDK1 activity phosphorylating cPKCβI would be not modulated by the BDNF/TrkB signalling pathway. On the other hand, muscle contraction induces the synthesis of cPKCβI, increasing total protein levels in both cytosol and membrane fractions. As described above, cPKCβI levels are enhanced by muscle contraction through the BDNF/TrkB signalling suggesting a retrograde regulation of this isoform. However, PDK1 levels and pPDK1 activity is maintained after

muscle contraction, suggesting that pcPKCβI is enhanced due to the increased amount of total cPKCβI caused by muscle contraction.

cPKCβI is directly involved in neurotransmission at the neuromuscular junction

After deepening the understanding of the mechanisms that maturate, activate and compartmentalize cPKCβI, it is important to determine its physiological functions. PKC is an important family of kinases that are usually involved in the modulation of neurotransmission since ACh increases when PKC is highly activated by PMA (Hori et al., 1999; Obis et al., 2015b; Santafé et al., 2005, 2006). The block of all PKC isoforms with CaC reduces approximately 40% of the evoked release in stimulated muscles, suggesting the involvement of PKC in neurotransmission. But PKC is usually uncoupled from ACh release mechanism at rest conditions because CaC, a potent inhibitor of PKC, is not able to reduce release in these conditions (Santafé et al., 2006). Nevertheless, PKC couples to ACh release when continuous electrical stimulation imposes moderate activity on the NMJ (Besalduch et al. 2010). Moreover, there is also functional evidence indicating that there is an interaction between TrkB receptors and PKC to modulate neurotransmission (Santafé et al., 2014). Considering all these evidence, **could be the presynaptic cPKCβI an isoform involved in the modulation of neurotransmission process?**

It has been suggested that at least one calcium-dependent PKC isoform might be involved in modulating ACh release in conditions in which PKC is active. That is because an increase of external calcium increases EPP size; this effect may be due partly to the involvement of the coupling of PKC to neurotransmission, because in the presence of high calcium, the blocking of the kinase with CaC reduces evoked release. These results suggest that the process could be mediated by a calcium-dependent PKC (Lanuza et al., 2014; Santafé et al., 2005). However, it is unknown which isoform of cPKC is involved in this regulation. In this thesis, we demonstrate that cPKCβI is directly involved in neurotransmission when a presynaptic stimulus is applied, enhancing ACh release at the NMJ. So far, previous results in our laboratory showed that nPKCε, another exclusively presynaptic PKC, is clearly

involved to maintain or potentiate ACh release in the NMJ but only in several conditions. For example, under stimulation conditions, its specific inhibitor is not able to decrease ACh release but after nPKCε inhibition, PKCs cannot be stimulated with PMA (Obis et al., 2015). These results suggest an indirect involvement of nPKCε helping transmitter release, probably by controlling the coupling of other PKC isoforms to the ACh release. In addition, PKC interacts with mAChRs and PKA to modulate neurotransmission in the NMJ, suggesting a complex and extensive mechanism of ACh release regulation (Santafé et al., 2006, 2009). Here we demonstrate that cPKCβI is directly linked to ACh release increasing neurotransmission and being a key factor in the maintenance of NMJ function. Thus, all mechanisms involved in cPKCβI synthesis, maturation, phosphorylation and activation have as one of their goals, modulate the neurotransmission process in the NMJ.

CONCLUDING REMARKS

The overall results of this thesis regarding the Chapter I are summarized in Figure 19. Synaptic activity and muscle contraction develop critical and coordinated roles to determine the complex modulation of cPKCβI by PDK1 and BDNF/TrkB at the NMJ:

Synaptic activity is involved in two important steps in the regulation of PKC function: priming of PKC by PDK1 and activation of PKC through BDNF/TrkB. Regarding the priming mechanism, synaptic activity translocates pPDK1 to the membrane fraction (#1) to (#2) increase the phosphorylation of cPKCβI in the same compartment. Once catalytically competent, pcPKCβI is activated through synaptic activity induced-BDNF/TrkB pathway (#3) to enhance ACh release.

Muscle contraction has a key role ensuring a pool of cPKCβI ready to be phosphorylated by PDK1. Consequently, muscle contraction further enhances the synaptic activity-induced increase of BDNF and decreases TrkB.T1 protein levels, ultimately increasing the ratio TrkB.FL/T1. It promotes the increase in total PKC protein levels (#4) in both cytosol and membrane, suggesting an activation of its synthesis or a decreasing of its degradation. This, results in an increased pool of total cPKCβI ready to be phosphorylated by PDK1, and then, activated by BDNF/TrkB to be involved in neurotransmission.

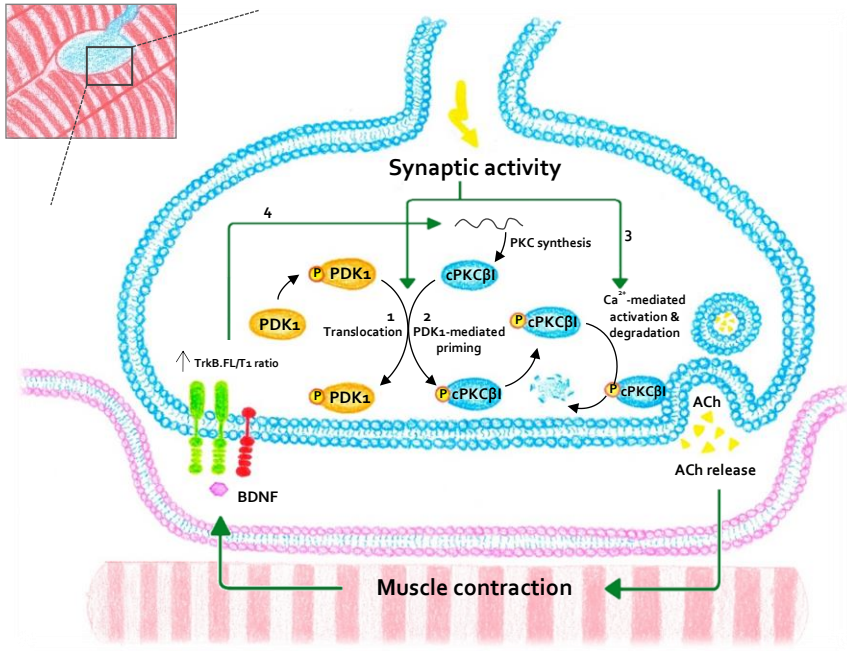

Figure 19. **Overview of cPKCβI regulation at the neuromuscular junction.**

The overall results of Chapter II show that in ALS context, where muscle contraction is decreased, BDNF signalling is impaired mainly due to the dysregulation of its receptors. mBDNF levels increase while $p75^{NTR}$, TrkB.FL, pTrkB.FL levels decrease and TrkB.T1 levels increase. However, when different

physical exercise is imposed in ALS mice (running or swimming-based training), and muscle contraction is increased, the impairment of BDNF signalling is prevented in a different way depending on the nature and intensity of the exercise. Future experiments are needed to understand how impaired BDNF/TrkB pathway can impact on PKCs and PDK1 function in ALS disease.

Thus, in this thesis we demonstrate that both synaptic activity and muscle contraction are closely coordinated and regulated in a complex and balanced way to preserve NMJ function. Further basic research is needed to understand the way that NMJ works and why it matters. It will provide the foundation of knowledge for the applied clinical science that follows. Thus, it will allow us a better understanding of neuromuscular disorders such as ALS, in which there is a progressive loss of the connection between nerve and muscle with an impaired NMJ function.

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F. CONCLUSIONS

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CONCLUSIONS

CHAPTER I

OBJECTIVE 1: To determine whether synaptic activity and muscle contraction affect proBDNF and mBDNF protein levels in the skeletal muscle.

- **Both synaptic activity and muscle contraction increase mature BDNF protein levels without altering proBDNF levels.**

OBJETIVE 2: To determine whether BDNF receptors TrkB.FL (and its phosphorylation), TrkB.T1 and $p75^{NTR}$ are modulated by synaptic activity and muscle contraction in the skeletal muscle.

- **Synaptic activity does not affect the protein levels of BDNF receptors.**
- **Muscle contraction reduces TrkB.T1 receptor without affecting TrkB.FL (thus, increasing TrkB FL/T1 ratio) or $P75^{NTR}$ protein levels.**
- **Both synaptic activity and muscle contraction differently modulate TrkB.FL phosphorylation at short times of activity. Synaptic activity increases TrkB.FL phosphorylation but muscle contraction decreases it. An effect declined back to near baseline after 30 minutes.**

OBJECTIVE 3: To determine whether cPKCα and cPKCβI (and their phosphorylation), are modulated by synaptic activity and muscle contraction in skeletal muscle. Moreover, to determine whether they are regulated by the activity-induced action of BDNF/TrkB signalling.

Synaptic activity:

- **Decreases cPKCα and cPKCβI total protein levels through BDNF/TrkB pathway, caused by an activation-induced degradation.**
- **Enhances cPKCα and cPKCβI phosphorylation but this mechanism is independent of BDNF/TrkB signalling.**

Muscle contraction:

- **Increases cPKCα and cPKCβI protein levels through an increase in TrkB FL/T1 ratio, by inhibiting PKC degradation or enhancing their synthesis. This may due to provide a pool ready to be activated.**
- **Consequently, enhances cPKCα and cPKCβI phosphorylation but this step is independent of BDNF/TrkB signalling.**

OBJECTIVE 4: To localize PDK1 in the NMJ.

- **PDK1 is located exclusively in the nerve terminal of the NMJ.**

OBJECTIVE 5: To determine whether PDK1 (and its phosphorylation) is modulated by synaptic activity and muscle contraction in skeletal muscle.

- **Neither PDK1 protein levels nor its phosphorylation are modulated by neuromuscular activity.**

OBJECTIVE 6: To specify whether PDK1 and cPKCβ are modulated by synaptic activity and muscle contraction in the cytosol and membrane fraction of skeletal muscle.

Synaptic activity:

- **Enhances pPDK1 translocation to the plasma membrane in parallel with an increase of its substrate pcPKCβI also in the membrane fraction.**

Muscle contraction:

- **Has not effect over PDK1 and pPDK1 levels. However, increases cPKCβI total protein in both cytosol and membrane fraction and pcPKCβI levels exclusively in the membrane fraction.**

OBJECTIVE 7: To determine the involvement of cPKCβI in neurotransmission.

- **Presynaptic cPKCβI enhances ACh release in skeletal muscle.**

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CHAPTER II

OBJECTIVE 1: To determine whether BDNF protein levels are affected in skeletal muscle of ALS mice (SOD1G93A) and whether they could be modulated by two different types of physical exercise (running and swimming-based training).

ALS mice (SOD1G93A):

- **mBDNF protein levels are increased while proBDNF levels are not affected.**

Trained ALS mice (SOD1G93A):

- **proBDNF levels are not modify by exercise.**
- **Both swimming and running protocols reverse the ALS-induced increase of mBDNF levels. However, only the swimming-based training efficiently preserves the mBDNF levels close to the control.**

OBJECTIVE 2: - To determine whether TrkB.FL (and its phosphorylation), TrkB.T1 and $p75^{NTR}$ are affected in skeletal muscle of ALS mice (SOD1G93A) and whether they could be modulated by two different types of physical exercise (running and swimming-based training).

ALS mice (SOD1G93A):

- **$p75^{NTR}$ protein levels are reduced.**
- **TrkB T1 protein levels are increased and TrkB.FL reduced, thus decreasing FL:T1 ratio.**
- **TrkB.FL phosphorylation levels are decreased.**

Trained ALS mice (SOD1G93A):

- **Only the running-based training efficiently preserves the $p75^{NTR}$ protein levels in ALS mice close to the control. $p75^{NTR}$ levels are not affected by the swimming-based training, thus keeping its protein levels decreased.**
- **Similarly, only the running-based training efficiently preserves the TrkB.T1 levels in ALS mice close to the control, not being affected by the swimming-based training, thus keeping its protein levels increased.**
- **Both training protocols efficiently preserve TrkB.FL protein levels close to the control.**
- **Both swimming and running protocols reverse the ALS-induced decrease of pTrkB. However, only the swimming-based training efficiently preserve the pTrkB levels close to the control.**

OBJECTIVE 3: To determine whether running and swimming-based training promotes different effects in ALS mice.

- **Swimming-based training is more efficient to preserve mBDNF and pTrkB levels close to the control mice. Whereas only running-based training maintains $p75^{NTR}$ and TrkB.T1 levels close to the control. Regarding TrkB.FL, both trainings are efficient to preserve TrkB.FL levels.**

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G. REFERENCES

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H. SCIENTIFIC CONTRIBUTIONS

UNIVERSITAT ROVIRA I VIRGILI
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Erica Hurtado Caballero

SCIENTIFIC CONTRIBUTIONS

Scientific articles belonging to this doctoral thesis:

Hurtado E, Cilleros V, Just L, Simó A, Nadal L, Tomàs M, Garcia N Lanuza MA, Tomàs J. (2017). "Synaptic activity and muscle contraction increases PDK1 and PKCβI phosphorylation in the presynaptic membrane of the neuromuscular junction". Frontiers in Molecular Neuroscience, 10:147. DOI: 10.3389/fnmol.2017.00270.

Hurtado E, Cilleros V, Nadal L, Simó A, Obis T, Garcia N, Santafé MM, Tomàs M, Halievski K, Jordan C, Lanuza MA, Tomàs J. (2017). "Muscle contraction regulates BDNF/TrkB signalling to retrogradely modulate synaptic function through presynaptic cPKCα and cPKCβI". Frontiers in Molecular Neuroscience, 10: 270. DOI: 10.3389/fnmol.2017.00147.

Other scientific articles:

Tomàs J, Garcia N, Lanuza MA, Nadal L, Tomàs M, **Hurtado E,** Cilleros V, Simó A. (2017). 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. Frontiers in Molecular Neuroscience. DOI: 10.3389/fnmol.2017.00255.

Tomàs J, Garcia N, Lanuza MA, Santafé MM, Tomàs M, Nadal L, **Hurtado E,** Simó A, Cilleros V. (2017). "Presynaptic membrane receptors modulate ACh release, axonal competition and synapse elimination during neuromuscular junction development". Frontiers in Molecular Neuroscience, DOI: 10.3389/fnmol.2017.00132.

Nadal L, Garcia N, **Hurtado E**, Simó A, Tomàs M, Lanuza MA, Cilleros V, Tomàs J. (2017). "Presynaptic muscarinic acetylcholine receptors and TrkB receptor cooperate in the elimination of redundant motor nerve terminals during development". Frontiers in Aging Neuroscience, 9:24. DOI: 10.3389/fnagi.2017.00024.

Nadal L, Garcia N, **Hurtado E**, Simó A, Tomàs M, Lanuza MA, Cilleros V, Tomàs J. (2017). "Synergistic action of presynaptic muscarinic acetylcholine receptors and adenosine receptors in developmental axonal competition at the neuromuscular junction". Developmental Neuroscience, 38(6):407-419. DOI: 10.1159/000458437.

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Obis T, Besalduch N, **Hurtado E**, Nadal L, Santafé MM, Garcia N, Tomàs M, Lanuza MA, Tomàs J. (2015). "The novel protein kinase C epsilon isoform at the adult neuromuscular synapse: Location, regulation by synaptic activity-dependent muscle contraction through TrkB signalling and coupling to ACh release". Molecular Brain, 8:8. DOI: 10.1186/s13041-015-0098-x.

Garcia N, Priego M, **Hurtado E**, Obis T, Santafé MM, Tomàs M, Besalduch N, Lanuza MA, Tomàs J. (2014). "Adenosine A2B and A3 receptors in the mice neuromuscular junction". Journal of Anatomy, 225(1):109-17. DOI: 10.1111/joa.12188.

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Participation in national and international congresses

Congress: 17th National Congress of the Spanish Society for Neuroscience (SENC).
Date and Place: 27-30 September 2017; Alicante (Spain).
Authors: Erica Hurtado, Laia Just, Víctor Cilleros, Anna Simó, Laura Nadal, Olivier Biondi, Frédéric Charbonnier, Neus Garcia, Maria Angel Lanuza, and Josep Tomàs.
Title: Physical exercise improves BDNF/TrkB/PKCβI signalling pathway in a mouse model of amyotrophic lateral sclerosis.
Format: Poster.

Congress: 17th National Congress of the Spanish Society for Neuroscience (SENC).
Date and Place: 27-30 September 2017; Alicante (Spain).
Authors: Víctor Cilleros, **Erica Hurtado**, Anna Simó, Laia Just, Laura Nadal, Manel Santafé, Marta Tomàs, Neus Garcia, Maria Angel Lanuza, Josep Tomàs.
Title: PKA and PKC isoforms are differentially modulated by M1 and M2 muscarinic autoreceptor subtypes to influence SNAP25 and Munc18-1 phosphorylation in the neuromuscular synapse.
Format: Poster.

Congress: 17th National Congress of the Spanish Society for Neuroscience (SENC).
Date and Place: 27-30 September 2017; Alicante (Spain).
Authors: Anna Simó, **Erica Hurtado**, Víctor Cilleros, Laura Nadal, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.
Title: nPKCε and cPKCβI modulate the synaptic activity induced phosphorylation of the exocytic protein MUNC18-1 at the adult neuromuscular junction.
Format: Poster.

Congress: X Symposium of Neurobiology.
Date and Place: 6-7 October 2016; Barcelona (Spain).
Authors: Erica Hurtado, Víctor Cilleros, Laura Nadal, Teresa Obis, Anna Simó, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.

Title: Synaptic activity-modulated BDNF-TrkB pathway enhances presynaptic cPKCβI to control neuromuscular synaptic function.

Format: Poster.

Congress: X Symposium of Neurobiology.

Date and Place: 6-7 October 2016; Barcelona (Spain).

Authors: Anna Simó, **Erica Hurtad**o, Víctor Cilleros, Laura Nadal, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.

Title: BDNF-TrkB-PKC signalling modulated by synaptic activity controls the phosphorylation of the exocytotic proteins Munc18-1 and SNAP25 at the neuromuscular junction.

Format: Poster.

Congress: X Symposium of Neurobiology.

Date and Place: 6-7 October 2016; Barcelona (Spain).

Authors: Víctor Cilleros, **Erica Hurtado**, Laura Nadal, Teresa Obis, Anna Simó, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.

Title: Synaptic activity-modulated BDNF-TrkB pathway enhances presynaptic cPKCβI to control neuromuscular synaptic function.

Format: Oral communication (Víctor Cilleros)

Congress: X Symposium of Neurobiology.

Date and Place: 6-7 October 2016; Barcelona (Spain).

Authors: Laura Nadal, Neus Garcia, **Erica Hurtado**, Anna Simó, Víctor Cilleros, Laia Just, Marta Tomàs, Maria Angel Lanuza, Manel Santafé, and Josep Tomàs.

Title: Adenosine receptors, mAChRs and TrkB modulate the developmental synapse elimination process at the neuromuscular junction.

Format: Poster.

Congress: 10th FENS Forum of Neuroscience.

Date and Place: 2-6 July 2016; Copenhagen (Denmark).

Authors: Erica Hurtado, Víctor Cilleros, Laura Nadal, Teresa Obis, Anna Simó, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.
Title: Synaptic activity-modulated BDNF-TrkB pathway enhances presynaptic cPKCβI to control neuromuscular synaptic function.
Format: Poster.

Congress: 10th FENS Forum of Neuroscience.
Date and Place: 2-6 July 2016; Copenhagen (Denmark).
Authors: Anna Simó, **Erica Hurtado**, Víctor Cilleros, Laura Nadal, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza and Josep Tomàs.
Title: BDNF-TrkB-PKC signalling modulated by synaptic activity controls the phosphorylation of the exocytotic proteins Munc18-1 and SNAP25 at the neuromuscular junction.
Format: Poster

Congress: 10th FENS Forum of Neuroscience.
Date and Place: 2-6 July 2016; Copenhagen (Denmark).
Authors: Laura Nadal, Neus Garcia, **Erica Hurtado**, Anna Simó, Víctor Cilleros, Marta Tomàs, Maria Angel Lanuza, Manel Santafé, and Josep Tomàs.
Title: Muscarinic acetylcholine autoreceptors, adenosine receptors and tropomyosin-related kinase B receptor (TrkB) cooperate in the developmental axonal loss and synapse elimination process at the neuromuscular junction.
Format: Poster.

Congress: Society for Neuroscience (SfN)
Date and Place: 17-21 October 2015; Chicago (USA).
Authors: Maria Angel Lanuza, **Erica Hurtado**, Laura Nadal, Teresa Obis, Anna Simó, Víctor Cilleros, Neus Garcia, Manel M. Santafé, Marta Tomàs, Josep Tomàs.
Title: BDNF and TrkB are regulated by both pre- and postsynaptic activity and enhance presynaptic cPKCβI to modulate neuromuscular synaptic function.
Format: Poster.

Congress: 16th National Congress of the Spanish Society for Neuroscience (SENC).

Date and Place: 23-25 September 2015; Granada (Spain).

Authors: Erica Hurtado, Laura Nadal, Teresa Obis, Anna Simó, Víctor Cilleros, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza, and Josep Tomàs.

Title: BDNF and TrkB are regulated by both pre- and postsynaptic activity and enhance presynaptic cPKCβI to modulate neuromuscular synaptic function.

Format: Poster.

Congress: 16th National Congress of the Spanish Society for Neuroscience (SENC).

Date and Place: 23-25 September 2015; Granada (Spain).

Authors: Anna Simó, **Erica Hurtado**, Laura Nadal, Víctor Cilleros, Neus Garcia, Manel Santafé, Marta Tomàs, Maria Angel Lanuza, and Josep Tomàs.

Title: Synaptic activity and PKC-TrkB signalling modulates the phosphorylation of the exocytotic proteins SNAP-25 and Munc18-1 at adult neuromuscular junction.

Format: Poster.

Congress: 16th National Congress of the Spanish Society for Neuroscience (SENC).

Date and Place: 23-25 September 2015; Granada (Spain).

Authors: Laura Nadal, Neus Garcia, **Erica Hurtado**, Anna Simó, Marta Tomàs, Maria Angel Lanuza, Manel Santafé, and Josep Tomàs.

Title: Presynaptic muscarinic acetylcholine autoreceptors (M_1, M_2 and M_4 subtypes) modulate the developmental synapse elimination process on the neuromuscular junction.

Format: Poster.

Congress: Society for Neuroscience (SfN)

Date and Place: 15-19 November 2014; Washington (USA).

Authors: Maria Angel Lanuza, **Erica Hurtado**, Nuria Besalduch, Teresa Obis, Laura Nadal, Neus Garcia, Manel M. Santafé, Mercedes Priego, Marta Tomàs, Josep Tomàs.

Title: The novel protein kinase C epsilon isoform at the neuromuscular synapse: location, synaptic activity-related expression, phosphorylation function and coupling to ACh release.

Format: Poster.

Congress: IX Symposium of Neurobiology.

Date and Place: 22-23 October 2014; Barcelona (Spain).

Authors: Erica Hurtado, Laura Nadal, Teresa Obis, Neus Garcia, Mercedes Priego, Manel Santafé, Marta Tomàs, Nicolàs Ortiz, Maria Angel Lanuza, Josep Tomàs.

Title: Blockade of tyrosine kinase receptor B prevents muscle contraction-induced presynaptic nPKCε, cPKCβI and cPKCα increases**.**

Format: Poster.

Congress: IX Symposium of Neurobiology.

Date and Place: 22-23 October 2014; Barcelona (Spain).

Authors: Laura Nadal, **Erica Hurtado**, Teresa Obis, Neus Garcia, Manel Santafé, Marta Tomàs, Mercedes Priego, Maria Angel Lanuza, Josep Tomàs.

Title: Activity-dependent changes of the nPKCε isoform through TrkB function in the adult rat neuromuscular synapse.

Format: Oral communication (Laura Nadal)

Congress: 9th FENS Forum of Neuroscience.

Date and Place: 5-9 July 2014; Milan (Italy).

Authors: Erica Hurtado, Teresa Obis, Mercedes Priego, Laura Nadal, Neus Garcia, Manel M. Santafé, Marta Tomàs, Nicolàs Ortiz, Maria Angel Lanuza and Josep Tomàs.

Title: Colocalization of protein kinase A (PKA) subunits and the A kinase anchoring proteins (AKAPS 9 and 150) in the neuromuscular synapse.

Format: Poster.

Congress: 9th FENS Forum of Neuroscience.

Date and Place: 5-9 July 2014; Milan (Italy).

Authors: Laura Nadal, **Erica Hurtado**, Teresa Obis, Neus Garcia, Mercedes Priego, Manel M. Santafé, Marta Tomàs, Nicolàs Ortiz, Maria Angel Lanuza and Josep Tomàs.

Title: Blockade of tyrosine kinase receptor B prevents muscle contraction-induced presynaptic nPKCε, cPKCβI and cPKCα increases.

Format: Poster.

Congress: 9th FENS Forum of Neuroscience.

Date and Place: 5-9 July 2014; Milan (Italy).

Authors: Teresa Obis, Núria Besalduch, Manel M. Santafé, Marta Tomàs, Neus Garcia, Mercedes Priego, **Erica Hurtado**, Laura Nadal, Maria Angel Lanuza and Josep Tomàs.

Title: Activity-dependent changes of the novel protein kinase C epsilon isoform in the neuromuscular synapse and its coupling to ACh release.

Format: Poster.

Congress: 9th FENS Forum of Neuroscience.

Date and Place: 5-9 July 2014; Milan (Italy).

Authors: Manel M. Santafé, Mercedes Priego, Teresa Obis, Neus Garcia, Marta Tomàs, Maria Angel Lanuza, Nicolàs Ortiz, **Erica Hurtado**, Laura Nadal and Josep Tomàs.

Title: Purinergic adenosine receptors and cholinergic muscarinic receptors cooperate in acetylcholine release modulation on neuromuscular synapse.

Format: Poster.

Congress: 15th National Congress of the Spanish Society for Neuroscience (SENC).

Date and Place: 25-27 September 2013; Oviedo (Spain).

Authors: Erica Hurtado, Neus Garcia, Manel Santafé, Mercedes Priego, Teresa Obis, Marta Tomàs, Núria Besalduch, Maria Angel Lanuza, and Josep Tomàs.

Title: Adenosine A2B and A3 receptors in the mice neuromuscular junction

Format: Poster.

Congress: 15th National Congress of the Spanish Society for Neuroscience (SENC).

Date and Place: 25-27 September 2013; Oviedo (Spain).

Authors: Teresa Obis, Mercedes Prieto, Manel Santafé, Neus Garcia, Maria Angel Lanuza, Marta Tomàs, Núria Besalduch, **Erica Hurtado** and Josep Tomàs.

Title: Involvement of serine kinases in the modulation of ACh release in the neuromuscular synapse

Format: Poster.

Congress: 15th National Congress of the Spanish Society for Neuroscience (SENC).

Date and Place: 25-27 September 2013; Oviedo (Spain).

Authors: Mercedes Prieto, Teresa Obis, Manel Santafé, Neus Garcia, Maria Angel Lanuza, Marta Tomàs, Núria Besalduch, **Erica Hurtado** and Josep Tomàs.

Title: Presynaptic membrane receptors in acetylcholine release modulation on neuromuscular synapse

Format: Poster.

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COORDINATED EFFECTS OF SYNAPTIC ACTIVITY AND MUSCLE CONTRACTION ON CPKC REGULATION BY PDK1 AND BDNF/TRKB SIGNALLING
Erica Hurtado Caballero

Mobility

Institution: Neuromuscular degeneration and plasticity (Team 9). Toxicology, Pharmacology and Cell Signalling. Paris Descartes University.

Supervisors: Dr. Olivier Biondi and Prof. Frédéric Charbonnier.

Project: Effects of physical exercise in a mouse model of Amyotrophic Lateral Sclerosis: role of PKCβI.

Length: 4 months (March-July 2017).

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