

A Neuron-Specific Protein found in Skeletal Muscle: New Frontiers for GAP-43

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*A Neuron-Specific Protein found in Skeletal Muscle:
New Frontiers for GAP-43*

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Preface

At the end of September 2005, I entered the Ce.S.I. Cell Physiology lab, University of Chieti “G.d’Annunzio” for the first time, as a Pharmacy pre-graduation student. That was my first step into biomedical scientific research and my acknowledgments start from that moment.

I believe my scientific path has been extremely constructive, under all points of view, with ups and downs, exactly like in ordinary life. Every day spent in the lab represented a new puzzle piece in my vocational training, from the scientific and the human perspectives.

*People I want to thank are countless. They all contributed and participated to my life during my graduate school. First of all, I give thanks to my unit components: Prof. Dora Marigliò, my supervisor, for teaching me to organize my experimental time and the meaning of working in a team, Dr. Simone Guarnieri, my tutor, for his unflagging kindness and patience through which he coached me, Dr. Katia Morabito who, firstly as a tutor and later as a colleague, meticulously supported me every time I needed help, and Dr. Silvio Sacchetti, trusted friend and crucial support during my *Xenopus Laevis* embryo crio-sectioning technique approach. A special thanks is addressed to Prof. Giorgio Fanò, department director and PhD coordinator, who gave me the opportunity of taking part in this superlative experience. I express my gratitude to Prof. Feliciano Protasi’s unit, notably to Dr. Cecilia Paolini for her precious advices and constant availability in the lab, and to Dr. Marco Dainese, always ready to provide new animals for experiments. A particular acknowledgment is reserved to Prof. Cinzia Volonté, my referee, who spent a considerable amount of time revising and commenting annual reports I used to send her at the end of each academic year. Besides, I thank Prof. Stefania Angelucci and Drs. Fabrizio Di*

Giuseppe and Marilisa Sulpizio, for the patient collaboration and the crucial data on bi-dimensional Western Blot analysis. A sincere thanks is for Prof. Nacira Tabti, ex-responsible for the neurophysiology INSERM 546 unit at the faculty of Medicine of University Pitié Saplétrière, in Paris, who kindly hosted and formed me in her lab for more than nine months, as well as for Dr. Thomas Carle and lab technician Francine Moueza, who supported me during my French stay with immense helpfulness. The project “GAP-43” has been conceived thanks to all of them.

An extraordinary thanks is moreover addressed to all who stood close to me before and during my PhD period, sharing my joys and sorrows: Ashley, my girlfriend, to have sustained me for over one year and half and finally helped me in the publication process of my dissertation; Christian, my precious fraternal friend, to have lived with me all the best and the worst adventures; Silvio, Oreste, Damiano and Peppe, to have participated to my daily life in Benevento; Felice and Domenico, to have made me laugh during the long days in the lab; my sister Maria Teresa, to always have understood my point of view and hosted me so many times in her apartment in Rome; my parents Nietta and Gigi, as well as my grandmother Tanina, who constantly have believed in my capabilities, by supporting and trusting me in all my decisions. I dedicate this dissertation work to all of them.

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INTRODUCTION

1. GAP-43

Growth Associated Protein 43 (GAP-43), a protein originally isolated from the synaptosomal plasma membranes of rat brain, and identified as a B-50 phosphoprotein band, was recognized and obtained from rat cerebral cortices 28 years ago (Oestreicher *et al.*, 1981; Oestreicher *et al.*, 1982). It is strictly implicated in neuronal development, growth cone guidance, cytoskeleton accretion, growth cone protrusion, neurite branching, cytoskeleton dissociation, growth cone shrinkage, cell death control and neuronal protection (Mosevitsky, 2005). It has been demonstrated that this protein is involved in cell growth regulation and regeneration, differentiation sequences, neurotransmitter release and synaptic plasticity (Apel & Storm, 1992). Moreover, it has been defined as “signal” protein because is involved in the major brain processes modulation. It has been localized mostly in axon terminals and growth cones of neuronal cells (presynaptic district of synapses) and growth cones (Burry *et al.*, 1992; Strittmatter *et al.*, 1995; Dent & Meiri, 1998). Its maximal expression has been observed during neurite outgrowth and neuronal regeneration after injuries (Dent & Meiri, 1998; Korshunova *et al.*, 2007). It has been estimated that a GAP-43 over-expression in the nervous system can enhance neurite sprouting, while a decrease of this protein concentration can lead to growth cone morphology alterations (Kapfhammer, 1997). In addition, it has been evaluated that in a neurodegenerative state, like Alzheimer’s disease (AD), its expression and function can result altered (Chakravarthy *et al.*, 2008). GAP-43 interactions are responsible of membrane rearrangements, necessary for triggering synaptic functions in mature brain (Coggins

& Zwiers, 1991; Benowitz & Routtenberg, 1997; Oestreicher *et al.*, 1997; Aarts *et al.*, 1999; Mosevitsky, 2005), where it interacts with cytoskeletal and signalling molecules, as like as calmodulin, actin and G-proteins (Coggins & Zwiers, 1991; Aarts *et al.*, 1999) and plays a fundamental role in neurotransmitter release, endocytosis, and long-term potentiation (LTP) (Neve *et al.*, 1998; Routtenberg *et al.*, 2000). GAP-43 has been shown to play a fundamental role in learning and memory by using knockout mice (Rekart *et al.*, 2005). A GAP-43 over-expression has been demonstrated to increase both learning and dentate gyrus during LTP. This effect was not observed with the GAP-43 protein kinase C (PKC) phosphorylation site mutant form. GAP-43 can bidirectionally modulate learning and memory in vivo and its PKC phosphorylation of presynaptic GAP-43 is essential for normal learning (Routtenberg *et al.*, 2000). Haruta and colleagues suggested that GAP-43 could directly affect the synaptic release machinery, like proteins SNAP-25, synaptobrevin and syntaxin (from the SNARE complex) or synaptotagmin, and furthermore this interaction is strictly dependent on the protein PKC phosphorylation (Haruta *et al.*, 1997). Other groups found that the GAP-43 phosphorylation process significantly influenced the neurotransmitter trafficking, thus increasing the released amount after phosphorylation (Dekker *et al.*, 1990; Heemskerk *et al.*, 1990) or decreasing it after GAP-43 reduction (Ivins *et al.*, 1993; Hens *et al.*, 1995). Briefly, GAP-43 presynaptic effects improve plasticity and memory. Another possible mechanism is the involvement of GAP-43 in synaptic development, which regulates plasticity and memory via developmental effects (Powell, 2006).

GAP-43 gene is of about 50 kb and located on human chromosome 3 and on mouse chromosome 16 (Grabczyk *et al.*, 1990).

Depending on the species, GAP-43 mRNA is translated into a protein of 194–238 aminoacids, and characterized by an anomalous electrophoretic mobility on SDS polyacrylamide gels. The protein size, seen in rat, is 23.6 kDa. GAP-43 migrates

within an apparent size of 40–60 kDa, depending on gel composition (Oestreicher *et al.*, 1984; Nishizawa, 1994).

The protein is subjected to several post-translational regulatory mechanisms that allow it to be in a cytosolic form or attached to membranes throughout dual palmitoylation sequences on cysteines 3 and 4 (Skene & Virag, 1989), or the myristoylation on N-terminal fragments, that requires the presence of a cysteine residue nearby (Alland *et al.*, 1994).

Additionally, GAP-43 represents one of the substrates for PKC that phosphorylates it on ser-41. In transgenic mice over expressing stable GAP-43 phosphorylated on ser-41, an increase of neuron sprouting in hippocampus region and in neuromuscular junction, is observed demonstrating that PKC activation is crucial for GAP-43 effect on neurite outgrowth (Fig. 1) (Aigner *et al.*, 1995). GAP-43 plays an important role also in the regulation of m-calpain activity in neurons, in axonal guidance and apoptosis (Zakharov & Mosevitsky, 2007).

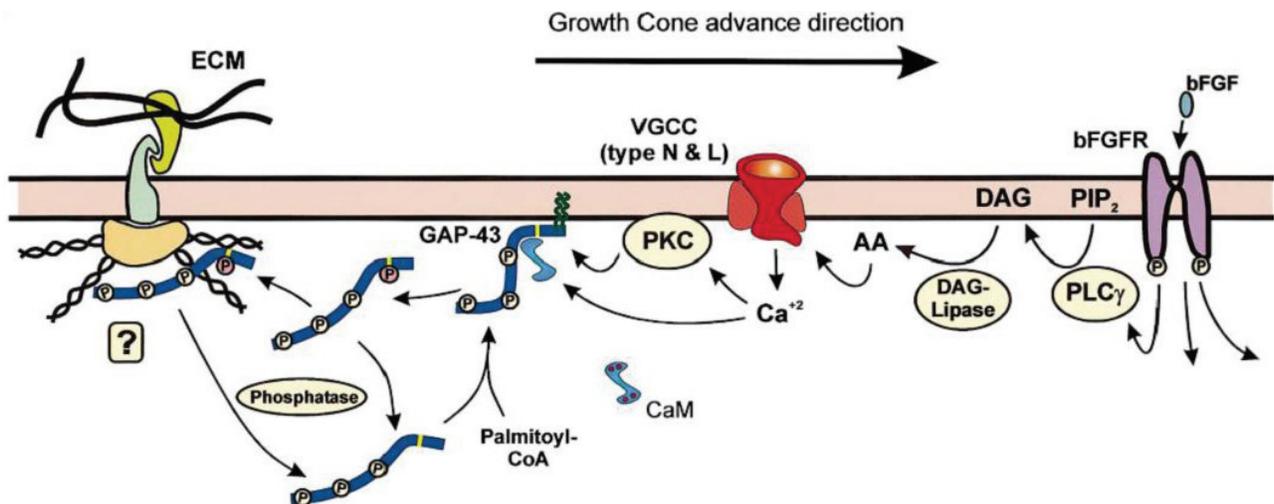


Figure 1. GAP-43 constitutes a primary substrate for protein PKC phosphorylation on ser-41.

GAP-43 protein presents two main structural domains: IQ domain and neuromodulin (Fig 2). The IQ domain binds calmodulin (CaM) and consequently may participate to intracellular calcium handling. Interestingly, the affinity of CaM to GAP-43 is independent on Ca^{2+} concentration and, in the absence of Ca^{2+} , CaM stabilizes an amphiphilic alpha-helical structure in GAP-43 affecting the interaction of the domain with PKC and plasma membrane (Gerendasy *et al.*, 1995; Hayashi *et al.*, 1997). This Ca^{2+} -independent CaM-binding to IQ domains was found in a variety of proteins, including brain-specific PKC substrate neurogranin108 and specific isoforms of myosin (Espreafico *et al.*, 1992). It has been suggested that GAP-43 may serve as a CaM “sponge” in the nerve-terminals, sequestering CaM near the membrane and releasing it in response to second messengers that result in GAP-43 phosphorylation (Skene & Virag, 1989).



Figure 2. GAP-43 is composed by two main structural domains:
an IQ domain and neuromodulin

1 10 20 30 40
MLC₃C₄MRRTKQVEKNDDDDQKIEQDGIKPEDKAHKAATK₃₇IQAS₄₁FRGHI
 50 60 70 80 90
TRKCLK₅₂GEKKDDVQAAEAEANKKDEAPVADGVEKKGEGTTTAEAAPAT
 100 110 120 130 140
GSKPDEPGKAGETPSEEKKGEGDAATEQAAPQAPASSEEKAGSAETESAT
 150 160 170 180 190
KASTDNSPSSKAEDAPAKEEPKQADVPAAVTAAATTPAEDAAAKATAQ
 200 210 220 230 238
PPTETGESSQAEENIEAVDETKPKESARQDEGKEEPEADQEHA

Figure 3. GAP-43 sequence (Homo sapiens), with relative palmytoilation (**Cys₃** and **Cy₄**) and PKC phosphorylation (**Ser₄₁**) sites, as well as the binding site for calmodulin (**IQ domain**) and the **neuromodulin domain**.

GAP-43 may block microfilament elongation after its binding with actin. Moreover, GAP-43 is strongly affected by calcium: it is able to bind apocalmodulin but this association can be inverted if calcium binds calmodulin. In addition, GAP-43 is the main neuronal target of PKC and its phosphorylation may cause its incapacity to bind calmodulin or capping actin filaments (He *et al.*, 1997). Local phosphorylation of GAP-43 is responsible for actin polymerization and local outgrowth. In fact, growth cones present high levels of the phosphorylated form of GAP-43 during the development (Dent & Meiri, 1998). Furthermore, inhibitory levels of cytosolic calcium exert their effect via calcineurin mediated dephosphorylation of GAP43 (Lautermilch & Spitzer, 2000).

To date, it is clearly demonstrated that GAP-43 in the nervous system is one of the first actors in the control of neurite outgrowth during development and neuronal regeneration after injuries (Dent & Meiri, 1998; Korshunova *et al.*, 2007). This data were also confirmed in GAP-43 knockout mice, in which the cerebellum appeared considerably reduced in dimensions, due to the neuronal precursor pool incapacity to spread out and thus showing that the GAP-43 gene knock-out can cause severe

abnormalities in axonal pathfinding and lead to high rate mice lethality (90–95%) within 2 weeks after birth (Strittmatter *et al.*, 1995; Zhang *et al.*, 2000; Shen *et al.*, 2002). Moreover, GAP-43 is also responsible for membrane rearrangements, necessary for triggering synaptic functions, in mature brain. At synaptic level, it interacts with cytoskeletal and signaling molecules, as well as calmodulin, actin and G-proteins, and plays a fundamental role in neurotransmitter release, endocytosis and LTP (Benowitz & Routtenberg, 1997; Neve *et al.*, 1998; Mosevitsky, 2005).

Even though GAP-43 was classified as neuron-specific protein, some years ago different authors demonstrated that this protein could be synthesized by Schwann cells depending on their functional status and/or during the degenerative process of peripheral nerves, although its role remains still unidentified (Vitkovic *et al.*, 1988; da Cunha & Vitkovic, 1990; Deloulme *et al.*, 1990; Curtis *et al.*, 1992).

In 2005, Ma and Collaborators studied the functional recovery of neuromuscular junctions following an *in vivo* botulinum toxin A (BoNT-A) injection in rat gastrocnemius muscle blocking acetylcholine release, and weakening muscle. They observed that GAP-43 mRNA, isolated from the whole muscle, was slightly up-regulated after BoNT-A injection in respect to untreated muscles. The authors concluded that these results could be due to nerve sprouting and Schwann activation among the muscle fibers (Ma *et al.*, 2005). Two years later, the same group found that total GAP-43 mRNA was up-regulated following nerve transection, in an *in vitro* rat neuromuscular model (Ma *et al.*, 2007). Also in this case they speculated that these results were related to the presence of GAP-43 immunoreactive Schwann cells overlying the degenerating axon terminals. Furthermore, the immunoreactive GAP-43 cells presented elaborate processes branching from the endplate region into the perisynaptic zone. Thus, it was hypothesized that GAP-43 could play a role in cell shape changes induced by alterations in the membrane structure and participate in the processes regulated by Schwann cells when their contacts with axons are disrupted

(Ma *et al.*, 2007). Additional evidence that GAP-43 is not confined in the nervous system was reported by Stoker and collaborators in 1992. They demonstrated the presence of both GAP-43 mRNA and protein in embryonic chicken limb. In particular they found a transient immunoreactivity for GAP-43 in cells positive to meromyosin, a specific skeletal muscle marker, suggesting that GAP-43 could be expressed also in muscle cell lineage. Moreover, immunostaining patterns they found suggested that positive GAP-43 cells fell into three main categories. Some immunoreactive cells were located in the sparsely innervated distal portions of the limb, especially in the interdigital mesenchyme, other GAP-43 positive cells were found in the developing muscle masses in the zygopodial regions and a third population was detected surrounding in-growing nerves within the zygopodial region, but did not express detectable levels of meromyosin (Stocker *et al.*, 1992). In addition, Stocker tried to provide a developmental significance of GAP-43 transient expression starting correlating his findings with the evidence that GAP-43 mRNA had been first detected in hindlimbs (from E5 chicken embryos), approximately one day after motor and sensory axons first enter the limb (Hamburger, 1975; Landmesser & Morris, 1975; Landmesser, 1978; Honig, 1982). GAP-43 transcripts peak in the hindlimbs when the last growth cones of motor axons reach the developing hindlimb muscles and begin to ramify through (Tosney & Landmesser, 1985a, 1985b). This temporal correlation indicates that interactions between in-growing axons and mesenchymal cells may induce expression of GAP-43 in a subpopulation of limb mesenchymal cells, including some cells of the muscle lineage. This notion was supported by Stocker's observations that some of GAP-43-positive cells were localized in a halo surrounding in-growing nerves, as if responding to such a paracrine factor. On the other hand, the absence of nerves in the vicinity of GAP-43 positive cell populations in the distal, interdigital region of the limb suggested that other unidentified signals might also be involved in the regulation of GAP-43 expression in these cells.

Heuss and Collaborators also investigated the presence of GAP-43 (B-50) in N-CAM and vimentin (N-CAM and cytoskeletal protein vimentin are excellent markers for regenerating muscle) serial thin sections of human skeletal muscle biopsies from healthy muscles or from those affected by inflammatory or dystrophic myopathies. By light-microscopic evaluations, they found a strong presence of GAP-43 in satellite cells, myoblasts, myotubes and small fibers deriving from inflammatory myopathies and muscular dystrophy, supporting the hypothesis that this protein may play a role in differentiation and development of human skeletal muscle. In normal muscle and in biopsies of neurogenic muscular atrophy, however, no light-microscopically demonstrable B-50 staining was found (Heuss *et al.*, 1995).

Some years later, Heuss and Schlotzer-Schrehardt performed a subcellular localization investigation of phosphoprotein B-50 in regenerating human skeletal muscle fibers by electron immunohistochemistry. They found that phosphoprotein B-50 immunoreactivity was randomly distributed over the nuclear and perinuclear area of regenerating muscle fibers. Previously, phosphoprotein B-50 immunoreactivity had been identified through light-microscopy on the inner face of the sarcolemma in hypotrophic type 1 fibers, in congenital fiber type disproportion. This phosphoprotein (B-50) distribution in developmentally disordered myocytes suggested an analogy to the corresponding evidence found in growing axons. However, they did not find subsarcolemmal expression of B-50 in regenerating muscle fibers, and thus speculated that there was a difference in B-50 expression between regeneration of muscle fibers and developmentally retarded/immature myofibers. As a consequence, they proposed that phosphoprotein B-50 was inserted only in the extending sarcolemma according to the developmental stage. Their immuno-electron microscopy protocols revealed that GAP-43 was localized in small round undifferentiated cells, resembling to myoblasts, in the area of regenerating muscle fibers and associated with cytoplasmic matrix and endoplasmic reticulum (Heuss & Schlotzer-Schrehardt, 1998).

2. The Muscular Tissue: Structure and Function

Animal's life is characterized on movement, based on some specialized proteins' function. These proteins are able to convert adenosin-triphosphate (ATP) hydrolysis-derived free energy, thus enhancing a mutual replacement on filamentous structures working as platforms. Muscle contraction is the outcome of cyclic ATP-dependent interactions between the proteins *myosin II*, defined as “molecular engine”, and *actin*, functioning as a dynamic surface.

2.1 Muscle types

Muscle tissue consists of highly specialized fibers which main task is to generate the force for contraction. Based on structural and functional characteristics, it has been classified into three categories:

- striated skeletal, representing the voluntary element of muscle mass;
- striated cardiac, the unique involuntary component of heart;
- unstriated smooth, involuntary and constituent hollow organs.

All types of muscles show their common origin in the embryo, where mesodermal cells called myoblasts form somites (representing skeletal muscle origins) and cells that migrate to form the smooth and the cardiac muscles of the body, early in the development.

Innervations of all muscle types are functionally correlated to muscle fibers themselves. The striated muscle is so defined because of its appearance in optical microscopy: dark and light bands represent the regular contractile protein disposition on 2 overlapped filaments. In particular, the skeletal muscle is employed for immediate and rapid action or postural maintenance and is innervated by large-diameter motor nerves, which can rapidly recruit a large number of fibers to provide

the force required, whereas the cardiac muscle is regulated by the vegetative nervous system and constitutes a hollow organ, which striated cells are synchronized and contract spontaneously as a single unit. The skeletal muscle is composed by long-shaped cells linking two skeletal joint bones to which the muscle is attached and their contraction is modulated by the voluntary control. Cardiac muscle tissue forms the bulk of the heart wall and its contraction is not under conscious control, so representing the most self-regulating muscle type, as it sets independently its own beat rate alternating the systole and diastole phases, defining the cardiac rhythm, although this activity is subjected to extensive control of autonomic nerves.

On the other hand, smooth muscle, lacking in striations and responsible for blood vessels, gastro-intestinal tube and all other empty organs motility, shows automatic contractions in reaction to the stretching of the hollow organs it surrounds, and to the activity in both branches of the autonomic nervous system, hormones in the blood, and intrinsic slow waves of membrane depolarization and repolarization.

2.2 Striated Skeletal Muscle

Skeletal muscle tissue is named for its location: attached to bones. It is composed by long shaped cells, identified as muscle fibers and associated in groups, enforced by elastic envelopes. Each muscular fiber presents several nuclei distributed along the filaments, and is contained in a specific membrane (sarcolemma) including several myofibrils in its cytoplasm (sarcoplasm), thus forming the muscular contraction platform. The sarcoplasm encloses the endoplasmic reticulum and mitochondria as well as several necessary components for muscle function, and supports the cytoskeleton. The sarcoplasm also works as a stock for important cell reserves like glycogen (fuel for muscle cells) and myoglobine (oxygen carrier). The sarcolemma presents a large number of fine tubular invaginations (called transverse tubules or T-tubules) regularly distributed and penetrating into fibers. Sarcoplasmic reticulum (SR)

results developed in muscle fibers and encloses T-tubules so figuring out triads, specific structures necessary for nervous signals transit (action potential) and calcium release from the SR and so enhancing the excitation-contraction coupling mechanism. Myofibrils build a regular filamentous structure (myofilaments) which gives muscles a striated appearance if observed in microscopy (Fig 4).

Myofibrils represent skeletal muscle cell contractile elements and a repetitive contractile units chain, defined as sarcomere, composes each one of them. On each myofibril, dark (called “A”) and light (called “I”) bands regularly alternate. Each A band is cut in the middle by a lighter area (H zone). In the middle of I bands there is a darker region called Z strip and all sections included within two consecutive Z strips represent a sarcomere, the smallest muscle fiber contractile unit (Fig 4).

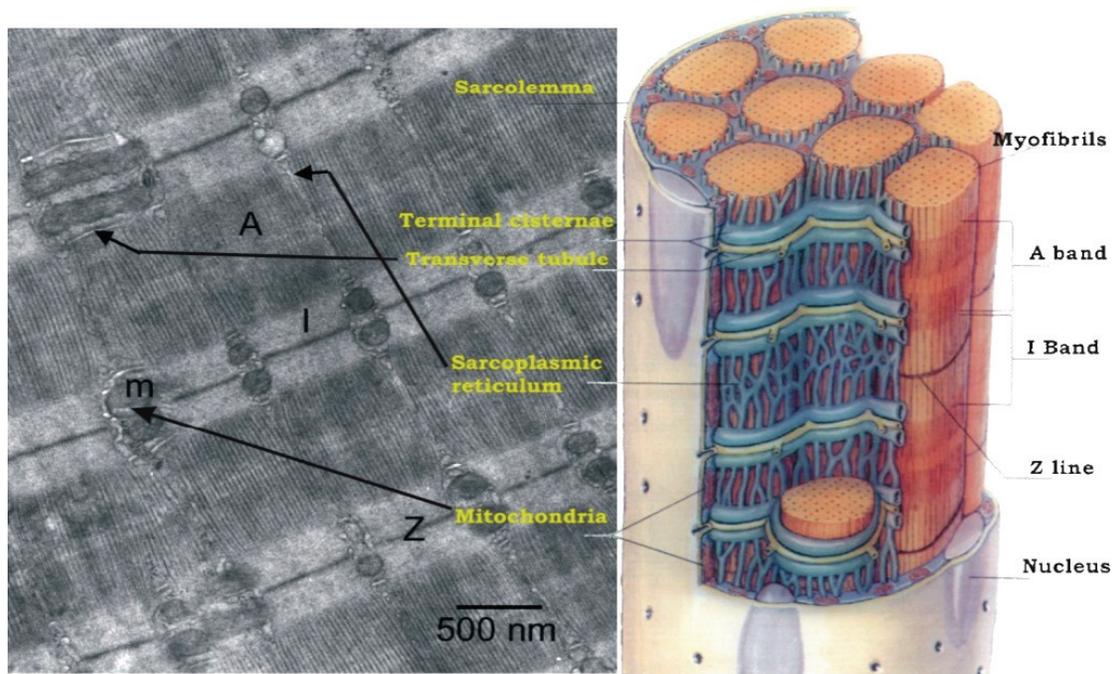


Figure 4. Skeletal muscle fiber ultrastructure (www.confolab.sav.sk).

From a molecular point of view, myofibrils strips originate from a regular disposition of the two myofilament types: thick filaments are composed by myosin molecules and thin ones by actin units. In order to sustain the metabolic consumption of muscles, sarcoplasm contains a large amount of mitochondria, which function is to obtain free energy from ATP hydrolysis, necessary for myofibril contraction. A single muscular fiber is the result of several undifferentiated (mononucleated) cells, called myoblasts, which undergo to fusion processes forming myotubes, presenting numerous nuclei in central position. In the end, during the differentiation phase from myotubes to muscle fiber, nuclei move towards muscle cells edge.

2.3 Calcium Release Units (CRUs): morphological characteristics

Some specialized domains, located at periodic intervals and related to the bands of myofibrils, either opposite the Z line or opposite the A-I junction, constitute the direct link between the muscle fiber plasmalemma and its core. Their connection has been demonstrated by electrophysiological data, measuring the quick electrical events occurring within the inner fiber. Junctional domains in the SR are associated with an outer membranes system (Fig. 5) figuring out well-defined junctions defined Calcium Release Units (CRUs) (Flucher & Franzini-Armstrong, 1996; Di Maio *et al.*, 2007).

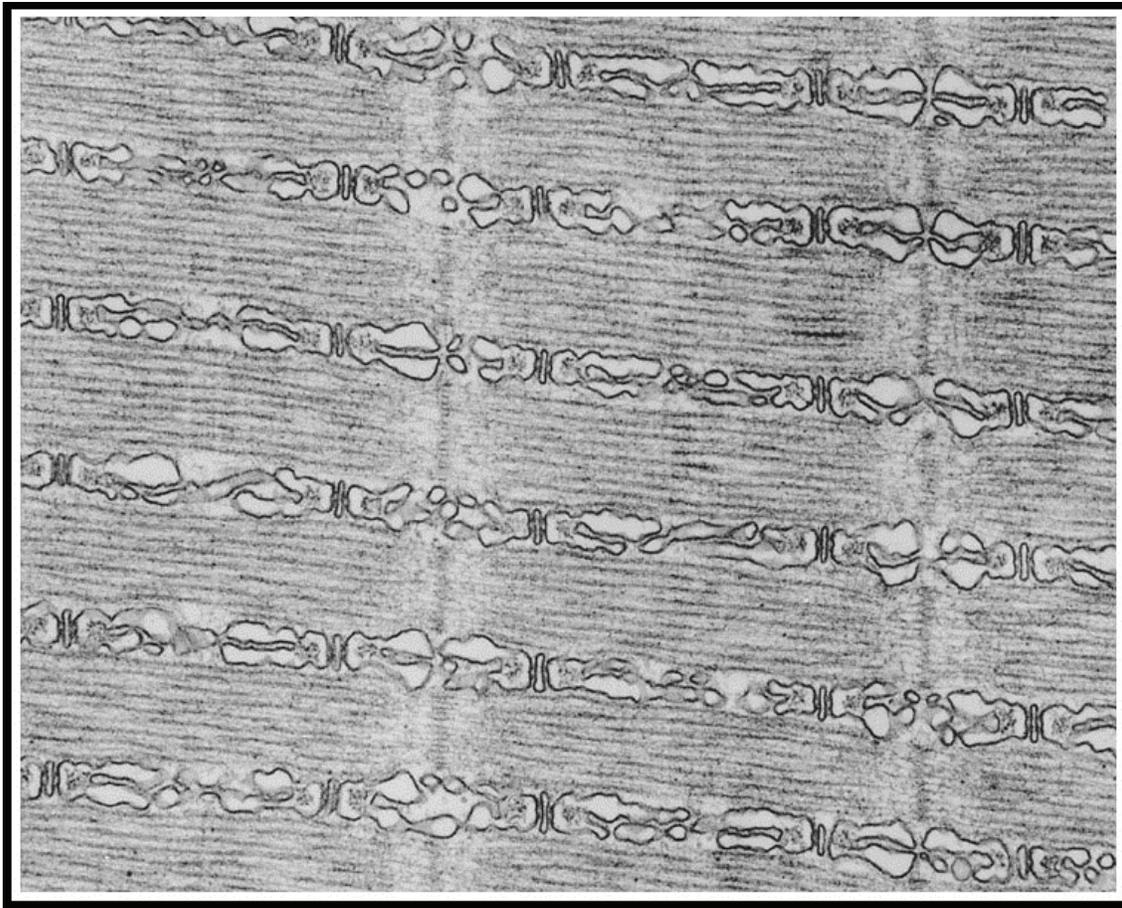


Figure 5. Longitudinal section oriented at right angles to long axis of T-tubule.

The junctional domains of the SR are tightly apposed, presenting a standardized 12nm width in the junctional gap, either to junctional domains of plasmalemma or to flat segments of T-tubules.

Three kinds of Calcium Release Units exist in muscle cells, depending on the nature and number of components involved (Fig 6):

- a) *Triads*, presenting a T-tubule tightly fixed between two jSR cisternae;
- b) *Dyads*, characterized by a junction formed between an SR cisterna and a T-tubule;
- c) *Peripheral couplings*, associated to the interaction between the surface membrane and the SR.

Despite triads, dyads and peripheral couplings show different conformations, they are all arranged by the same molecular and structural elements (Smith, 1966; Sommer *et al.*, 1991; Schneider, 1994; Flucher & Franzini-Armstrong, 1996; Di Maio *et al.*, 2007).

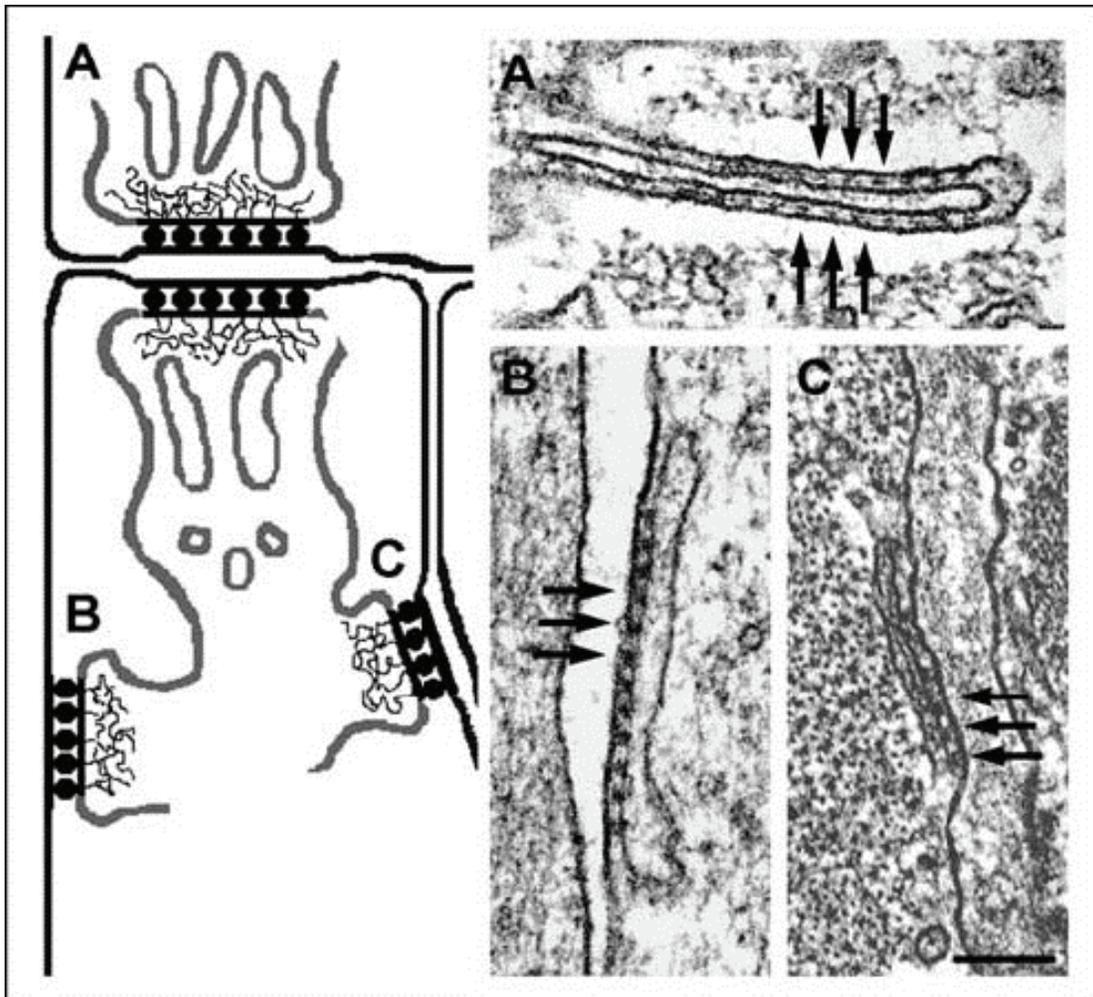


Figure 6. Examples of Calcium Release Units in muscle cells. A) Triads are composed by one T-tubule sided by two SR cisternae (from adult toadfish swimbladder muscle). B) Peripheral couplings are formed by SR vesicles and the surface membrane (from BC3H1 cells). C) Dyads are assembled by SR vesicles and T-tubules (from canine heart).

Triads are the only kind of junction present in adult skeletal muscle fibers, whereas dyads and peripheral couplings are the predominant type of CRUs in developing muscle and in cardiac muscle (Sun *et al.*, 1995; Flucher & Franzini-Armstrong, 1996).

2.4 Major components of Calcium Release Units

To date, several proteins of the jSR have been well described in terms of localization and function. The most expressed ones are the Ryanodine Receptors (RyRs), also defined as SR calcium release channels (Sutko & Airey, 1996), Calsequestrin (MacLennan & Wong, 1971; Meissner, 1975), Triadin (Caswell *et al.*, 1991) and Junctin (Mitchell *et al.*, 1988). Facing the SR, there is another crucial protein for calcium release, located on external membranes of junctional domains, called L-type calcium channel, also known as Dihydropyridine Receptor (DHPR).

Moreover, the skeletal muscle specific isoforms of two proteins mentioned above, RyR1 and α_{1S} DHPR, are structurally and functionally linked to each other within the apposed junctional domains of CRU (Block *et al.*, 1988; Takekura *et al.*, 1994; Protasi *et al.*, 1998).

2.5 Ryanodine Receptor

RyR is a high-molecular-weight ($\sim 2 \cdot 10^6$ Daltons) homotetramer composed of four identical 565 kDa subunits (Fig 7). Each subunit presents a short hydrophobic domain inserted in the jSR membrane, in order to constitute the channel region, and a large hydrophilic domain in the cytoplasm (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990).

The RyR cytoplasmic domain, composed by four subunits, has been called *foot* (Lai *et al.*, 1988). Then, the unusually large size and characteristic shape of the feet have allowed their direct identification with the large spanning proteins or Ryanodine

Receptor, RyR (Block *et al.*, 1988; Lai *et al.*, 1988) also known as channel responsible for release of calcium from the SR.

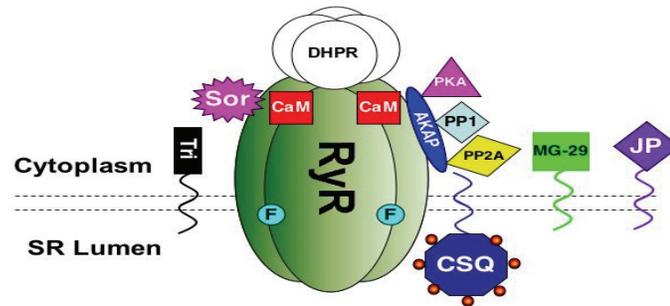


Figure 7. The ryanodine receptor (RyR) and its associated proteins (<http://www.bbri.org/people/fessenden.html>).

Thanks to the three-dimensional reconstruction of the RyR, from negatively stained and frozen hydrated specimens, it has been possible to study the complex interactions this molecule is able to perform. (Wagenknecht *et al.*, 1989; Sharma *et al.*, 1998)

Three types of RyR are known in literature and slightly differ each other from a structural point of view of the cytoplasmic domains (Fig. 8) (Wagenknecht *et al.*, 1989; Sharma *et al.*, 1998; Liu *et al.*, 2001).

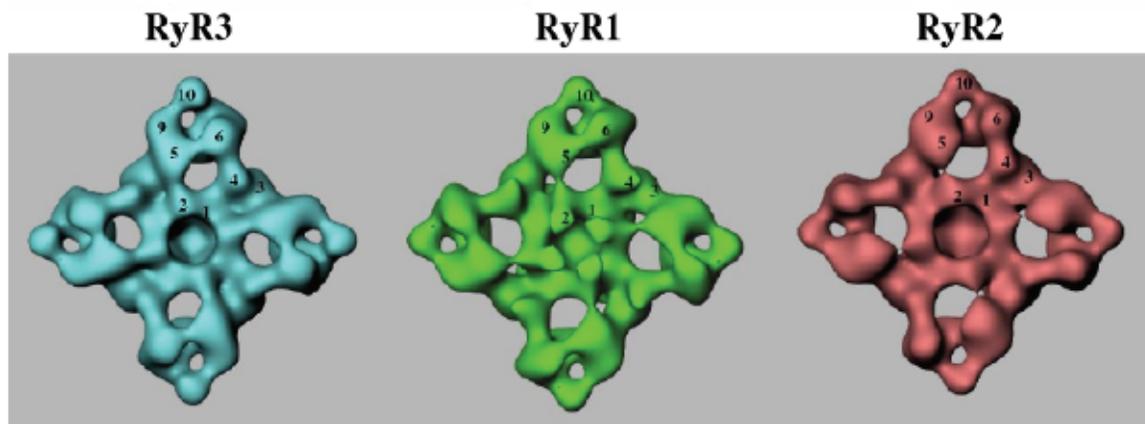


Figure 8. Comparison of 3D reconstructions of the three types of RyR viewed onto the cytoplasmic surface. Note conservation of domain architecture (Wagenknecht & Samsó, 2002).

RyR1 is the only RyR isoform able to link to DHPR and subsequently enhance the excitation-contraction (e-c) coupling in the skeletal muscle. RyR3 does not sustain e-c coupling in the absence of RyR1, (Takeshima *et al.*, 1989; Sutko & Airey, 1996; Protasi *et al.*, 2000) does not link to skeletal DHPR (Protasi *et al.*, 2000) and its absence in mouse has only minor effects on muscle activity (Barone *et al.*, 1998).

2.6 Dihydropyridine Receptor

The dihydropyridine receptor is a fundamental component of CRUs. It constitutes an L-type (slowly activating) Ca^{2+} channel, associated with depolarizations to the contractile threshold. It is specifically localized in areas of exterior/T-tubule membranes, facing the feet junctional disposition in skeletal and cardiac muscle fibers. It represents the voltage sensor of the e-c coupling mechanism (Jorgensen *et al.*, 1989; Carl *et al.*, 1995).

Several subunits contribute to DHPRs composition: the critical α_{1S} subunit and the auxiliary α_2 - δ , β , and γ subunits (Fig. 9) (Catterall, 2000). The gating machinery and the ion pore are located within the α_{1S} subunit.

The α_1 channel-forming subunit of the DHPR is also muscle type specific: the α_{1S} is present in skeletal muscle and the α_{1C} in cardiac muscle (Block *et al.*, 1988; Catterall, 1991). The two isoforms share ~66% homology, mostly confined to the transmembrane regions, while higher divergences are found in the cytoplasmic domains and the loops that are thought to interact with RyRs. The isoforms have different channel kinetic properties and interact differently with RyRs. The other four subunits are regulatory components of the channel.

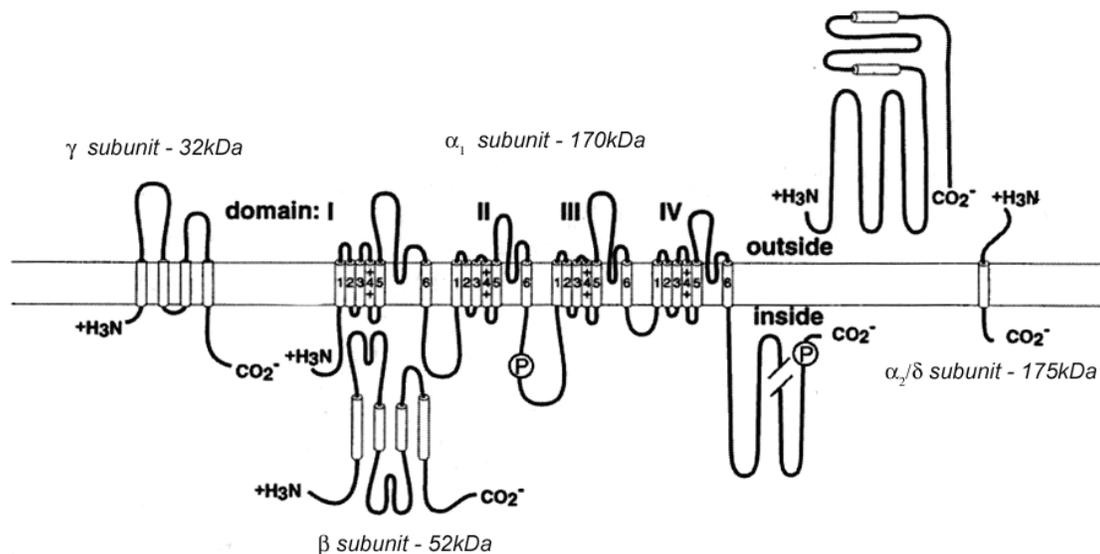


Figure 9. Model of L-type Ca^{2+} channel subunit structure (Takahashi *et al.*, 1987; Catterall, 2000).

DHPRs are identified as intramembraneous elements in the cytoplasmic leaflet of the surface membrane, and is directly in contact with the cytosol or facing the jSR membrane (Block *et al.*, 1988).

DHPRs may be organized in numerous dispositions, but in skeletal muscle are grouped by four units, representing the *tetrads* (Block *et al.*, 1988). The four DHPR components occupy four binding sites of one RyR1 (one for each equal RyR subunit), and the centers of the tetrads are located in the peripheral area of the centers of the feet subunit. Tetrads are situated roughly above the four identical RyR subunits and this feature allows the formation of the “clamp”, which changes configuration when the channel opens after the coupling between the voltage-gated Ca^{2+} channels, belonging to the T-tubule membrane, and the SR membrane Ca^{2+} -release channel.

The clamp-like RyR1 subdomains are located at a distance of $\sim 180 \text{ \AA}$ from each other, placed at the four corners in the square-shaped cytoplasmic region. This distance is consistent with the center-to-center spacing of the Ca^{2+} channels in the tetrads above RyR1, (Protasi *et al.*, 1997) making the clamps likely sites of this protein–protein coupling. These clamp-like domains also undergo significant conformational changes upon opening of the Ca^{2+} -release channel (Serysheva *et al.*, 1995; Nakai *et al.*, 1998).

2.7 Excitation contraction-coupling

In order to perform a fast and highly accurate contraction, the vertebrate skeletal muscle has developed an exclusive control apparatus for regulating the $[\text{Ca}^{2+}]_i$ which is able to modulate the force development by the contractile machinery.

The event enhancing muscle contraction is an increase of $[\text{Ca}^{2+}]_i$. At rest, this concentration is $\sim 0.1 \mu\text{mol} \times \text{L}^{-1}$, but during a stimulation may augment up to $0.1 \text{ mmol} \times \text{L}^{-1}$. The e-c coupling is strictly correlated to mechanisms allowing this $[\text{Ca}^{2+}]_i$ increase, due to the massive calcium ions release from the SR. An incoming action potential (AP) within the nerve terminal start the neuromodulator (acetylcholine) deliverance in the synaptic slit. After its diffusion in the intersynaptic space, acetylcholine binds to its specific receptor, the acetylcholine nicotinic receptor, a