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Preliminary study on sarcoglycan sub-complex in rat cerebral and cerebellar cortex

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Summary

The sarcoglycan sub-complex is a protein system which plays a key role in sarcolemma stabilization during muscle activity. Although numerous studies have been conducted on this system, there are few data about its localization in non-muscular tissues. On this basis we carried out an indirect immunofluorescence study on normal rat cerebral and cerebellar cortex. In particular, we carried out single localization reactions to analyze if these proteins are present in brain and double localization reactions between sarcoglycans and either SMI-32 or GFAP to verify if they are expressed both in neurons and glial cells. We found that all tested sarcoglycans are present both in cerebral and cerebellar cortex and that they are expressed both in neurons and glial cells. The typical staining pattern of all sarcoglycans is represented by "spot-like" fluorescence, with spots of 0.5-2 μm average diameter laid out mainly around the soma of the cells. The main difference about sarcoglycans expression between cerebral and cerebellar cortex is that in the cerebellar cortex the sarcoglycans positivity is detectable only in an area which is likely to correspond to Purkinje cells layer. The presence of sarcoglycans in cerebral and cerebellar cortex and their disposition mainly around the soma of the cells suggest a role of these proteins in cellular signalling and in regulating postsynaptic receptor assembly mainly in axo-somatic synapses.

Key words

SMI-32; GFAP; brain; cerebellum; immunofluorescence; neurons; neuroglia.

Introduction

The sarcoglycan sub-complex (SGC) is a protein system made up of transmembrane glycoproteins which creates a connection between extracellular matrix components and cytoskeleton; this protein system is a member of dystrophin glycoprotein complex which, besides sarcoglycans, contains the sarcoplasmic sub-complex, which includes dystrophin, dystrobrevin and syntrophins, and dystroglycan sub-complex which includes α- and β-dystroglycans.

The identification of sarcoglycans at first in muscle tissue suggests a key role of these proteins in sarcolemma stabilization during muscle activity (Revisit et al., 1990; Yoshida and Ozawa., 1990; Revisit and Campbell., 1991).

The SGC is made up of four transmembrane glycoproteins: one type I protein, α-sarcoglycan (50 kD), harbouring the N-terminal on the extracellular side, and three

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type II proteins, β-, γ- and δ-sarcoglycans (43 kD, 35 kD and 35 kD, respectively), harbouring the N-terminal on the intracellular side (Yoshida et al., 1994). This suggests that the sarcoglycans are divided in two different subunits: the first one, made up of α-sarcoglycan and the second one made up of β-, γ- and δ-sarcoglycans (Hack et al., 1998; Chan et al., 1998).

By molecular investigations, the way sarcoglycans link to each other to create the final complex has been studied. The results have shown that the assembly of the protein components starts from a central core, made up of β- and δ-sarcoglycans; after formation of β/δ-core, α- and γ-sarcoglycans determinate the maturation of the complex and the link to dystrophin (Hack et al., 2000).

The SGC, similar to dystrophin, has also been extensively studied because of its involvement in some forms of hereditary muscular dystrophy, sarcoglycanophaty or Limb Girdle Muscular Dystrophy (LGMD) (Roberds et al., 1994; Bönnemann et al., 1995; Lim et al., 1995; Betto et al., 1999). The sarcoglycans have been mainly studied in skeletal and cardiac muscle where they show a costameric distribution (Anastasi et al., 2003).

A fifth sarcoglycan, ε-sarcoglycan, highly homologous to α-sarcoglycan, has been identified (Ettinger et al., 1997). The ε-sarcoglycan, because of its ubiquitous expression, is not considered muscle-specific (Ettinger et al., 1997; Chan et al., 1998; Duclos et al., 1998).

Since the identification of ε-sarcoglycan, the studies about these proteins have also been extended to smooth muscle fibres where it was hypothesized that the ε-sarcoglycan might replace α-sarcoglycan (Straub et al.,1999).

A novel mammalian sarcoglycan, ζ-sarcoglycan, with significant homologies to γ- and δ-sarcoglycan, has been identified (Wheeler et al., 2002).

The identification of this sixth sarcoglycan, ζ-sarcoglycan, suggests the existence of two different tetrameric complexes: the first one, made up of α-, β-, γ- and δ-sarcoglycan, characteristic of cardiac and skeletal muscle, and the second one, made up of β-, δ-, ε- and ζ-sarcoglycan, characteristic of smooth muscle (Straub et al., 1999; Wheeler et al., 2003).

In opposition to these results, a study carried out by immunofluorescence on smooth muscle has shown the expression of all five sarcoglycans, α-, β-, γ-, δ- and ε-sarcoglycans in this tissue (Anastasia et al., 2005). The presence of ζ-sarcoglycan was confirmed by molecular techniques. These results suggest the intriguing possibility of the existence of a pentameric - or hexameric considering ζ-sarcoglycan - SGC, with a higher or lower expression of individual sarcoglycans depending on muscle type (Anastasia et al., 2007).

The SGC, for its functional and structural role in stabilization of muscular fibres, was for long time studied only in muscular tissues. There are few data about sarcoglycans in non-muscular tissues.

The study of sarcoglycans in non-muscular tissues has been mainly carried out on brain where recent researches have shown the importance of ε-sarcoglycan (DYT11) which, when mutated, determines Myoclonus Dystonia Syndrome (Zimprich et al., 2001). In the cerebellum ε-sarcoglycan was found in the Purkinje cell and the molecular layers (Chan et al., 2005). In addition, the most recently described member of the sarcoglycan family, ζ-sarcoglycan, is also highly expressed in brain (Shiga et al., 2006).

Therefore, on this basis, it is considered possible that the tetrameric ε-β-ζ-δ-complex be present in the brain. This complex has already been described in

Schwann cells of peripheral nerves (Cai et al., 2007). However, although theoretically possible, the existence of a prototypical tetrameric sarcoglycan complex in the brain is considered unlikely (Waite et al., 2009).

Our recent studies on human brain biopsies, carried out by immunofluorescence, have demonstrated the expression of all five sarcoglycans, α-β-γ-δ-ε (unpublished data). On this basis, in this study we wanted to verify if all is forms of sarcoglycans are expressed in cerebral and cerebellar cortex of the rat.

Materials and methods

In the present study, five normal male Westar rats were used. The animals were sacrificed after anaesthesia and then their brains were extracted and fixed in 3% paraformaldehyde in 0.2 mol/L phosphate buffer, pH 7.4. After numerous rinses in 0.2 mol/L phosphate buffer and phosphate buffered saline 0.2 mol/L, pH 7.6 with 0.9% Nail (PBS), they were infiltrated with 12% and 18% sucrose and then both the cerebrum and cerebellum were divided in two hemispheres.

The left hemisphere of cerebrum and cerebellum were frozen in liquid nitrogen and stored at -20° and reserved for sagittal cuts; the right hemispheres were sectioned by cryotomy, with coronal cuts in anterior to posterior direction, in 30 μm sections collected on glass slides coated with 0.5% gelatine and 0.005% chromium potassium sulphate. Three series of immunofluorescence reactions were performed: 1) single localization for α-, β-, γ-, δ-, and ε-sarcoglycans; 2) double localization between each sarcoglycans and SMI-32, a protein which marks a non-phosphorylated epitope of neurifilament proteins of neurons; 3) double localization between each sarcoglycans and GFAP, a protein which marks the glial fibrillary acidic protein of glial cells.

To block non-specific sites and to make membranes permeable, the sections were pre-incubated with 1% bovine serum albumin and 0.3% Triton X-100 in PBS at room temperature for 15 min and then with primary antibodies, at room temperature for 2h. The following primary antibodies, obtained from Santa Cruz Biotechnology Inc. (CA, USA), were used: rabbit polyclonal anti-α-sarcoglycan (diluted 1:100); goat polyclonal anti-β-sarcoglycans (diluted 1:100); goat polyclonal anti-γ-sarcoglycan (diluted 1:100); goat polyclonal anti-δ-sarcoglycan (diluted 1:100); goat polyclonal anti-ε-sarcoglycan (diluted 1:100). Rabbit polyclonal anti-α-sarcoglycan antibody was demonstrated with Texas Red-conjugated IgG anti rabbit (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and goat polyclonal anti β-, γ-, δ-, and ε-sarcoglycans antibodies were demonstrated with Texas Red-conjugated IgG anti goat (1:100 dilution; Jackson ImmunoResearch Laboratories); secondary antibodies were applied for 1 h at room temperature. For double localization reactions the sections were incubated also with the following primary antibodies, at room temperature for 2h; mouse monoclonal anti SMI-32 RT (1:1000 dilution; Covance, Eteryville, CA, USA) and mouse monoclonal anti GFAP (Santa Cruz Biotechnology, Inc., CA, USA). Fluorescein isothiocyanate (FITC)-labelled anti mouse antibody (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as secondary one for these antigens.

To label nuclei, after incubation with fluorochrome, the sections were incubated with DAPI (1:1000 dilution; Sigma Chemicals, St Louis, MO, USA) for 10 min.

Slides were finally washed in PBS and sealed with mounting medium. Samples were observed with a Zeiss LSM 510 confocal microscope equipped with Argon laser (458 nm and 488 nm λ) and two HeNe laser (543 nm and 633 nm λ). All images were digitized at a resolution of 8 bits into an array of 2048 x 2048 pixels. Optical sections of fluorescence specimens were obtained at 488 nm λ, at 62/s scanning speed with up to 8 repetitions on average. The pinhole size was set for optimal resolution. Contrast and brightness were established by examining the most brightly labelled pixels and choosing settings that allowed clear visualization of structural details while keeping the highest pixel intensities near 200. Digital images were cropped and figure montages were prepared using Adobe Photoshop 7.0.

Results

In order to verify the presence of sarcoglycans in cerebral and cerebellar cortex, single localization reactions for each sarcoglycans were performed. Observing cerebral cortex sections we found that a fluorescence pattern for α- (Fig. 1A), β- (Fig. 1B), γ- (Fig. 1C), δ- (Fig. 1D), ε-sarcoglycan (Fig. 1E) was detectable by red fluorescence.

Moreover, at higher magnification, it was shown that the staining pattern of α-sarcoglycan (red channel), as all of other sarcoglycans (data not shown), appeared

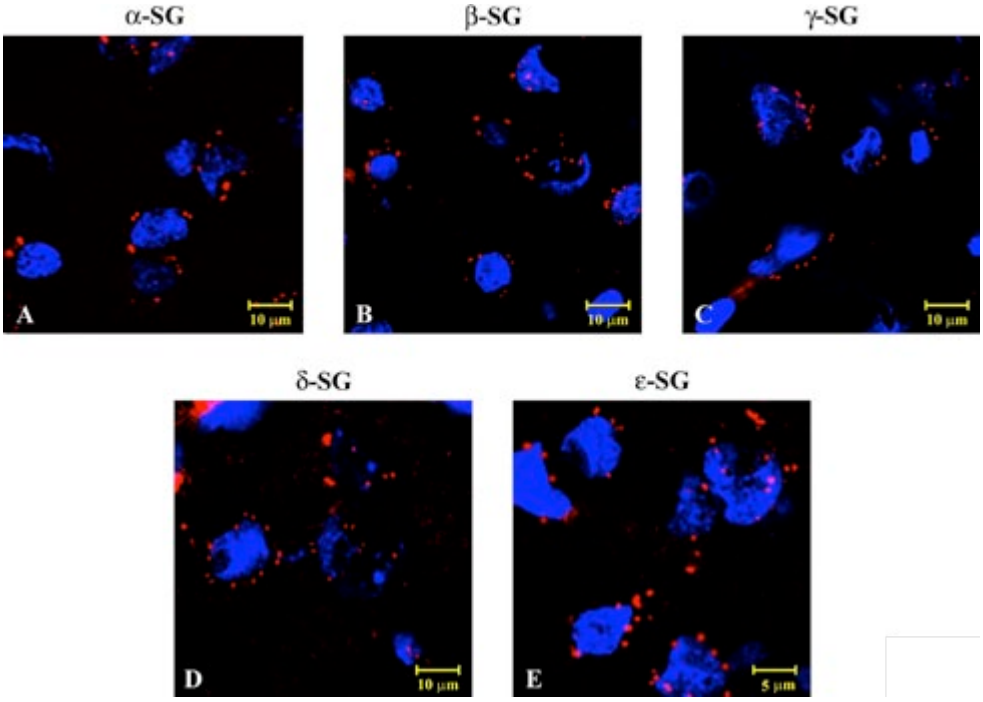

Figure 1 – Confocal image of rat cerebral cortex labelled with anti-α- (A), β- (B), γ- (C), δ- (D), and ε-sarcoglycans (E) (red channel) antibodies. The blue colour represents nuclear staining DAPI.

as "spot-like" fluorescence, with spots about of 0.5-2 μ, which were distributed all around the soma of neurons(Fig. 2) whereas in glial cells the labelling was distributed in the central portion of the cell (Figs. 3B and 6B).

To understand which type of cerebral cells express sarcoglycans, we carried out double labelling reactions using antibodies against β-sarcoglycan and SMI-32 (red and green channel respectively in Fig. 3A), which marks the big pyramidal neurons, and with antibodies against δ-sarcoglycan and GFAP (red and green channel respectively in Fig. 3B), which marks glial cells. SMI-32 and GFAP appeared also colocalized with all other sarcoglycans (data not shown). These double localizations showed that each sarcoglycan is expressed both in neurons and glial cells where it is possible to observe colocalization represented by points of yellow fluorescence by merging red and green fluorescence (Fig. 3 A,B).

Observing cerebellar cortex sections, we found labelling for α- (Fig. 4A), β- (Fig. 4B), γ- (Fig. 4C), δ- (Fig. 4D), ε-sarcoglycan (Fig. 4E) also in this region. Moreover, the γ-sarcoglycan staining pattern was represented with a "spot-like" pattern similar to

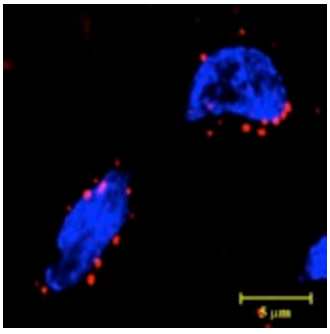

Figure 2 – Typical "spot-like" staining pattern of α-sarcoglycan (red channel) around the body of the cells, with spots about of 0.5-2 μ. The blue colour represents nuclear staining DAPI.

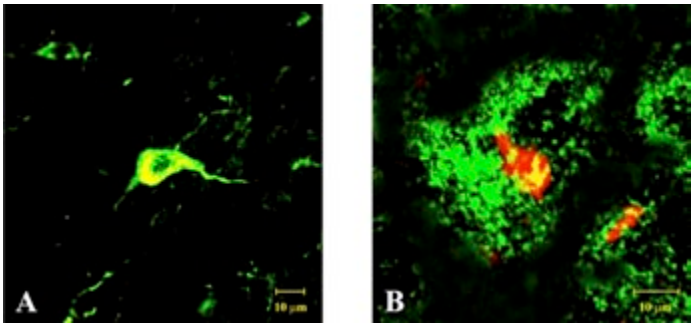

Figure 3 – Confocal image of rat cerebral cortex double-labelled with anti-β-sarcoglycan (red channel) and anti-SMI-32 (green channel) antibodies; the fluorescence appears as spot-like (A). Double-labelling with anti-δ-sarcoglycan (red channel) and anti-GFAP (green channel) antibodies; the fluorescence is distributed in the central portion of the cell (B).

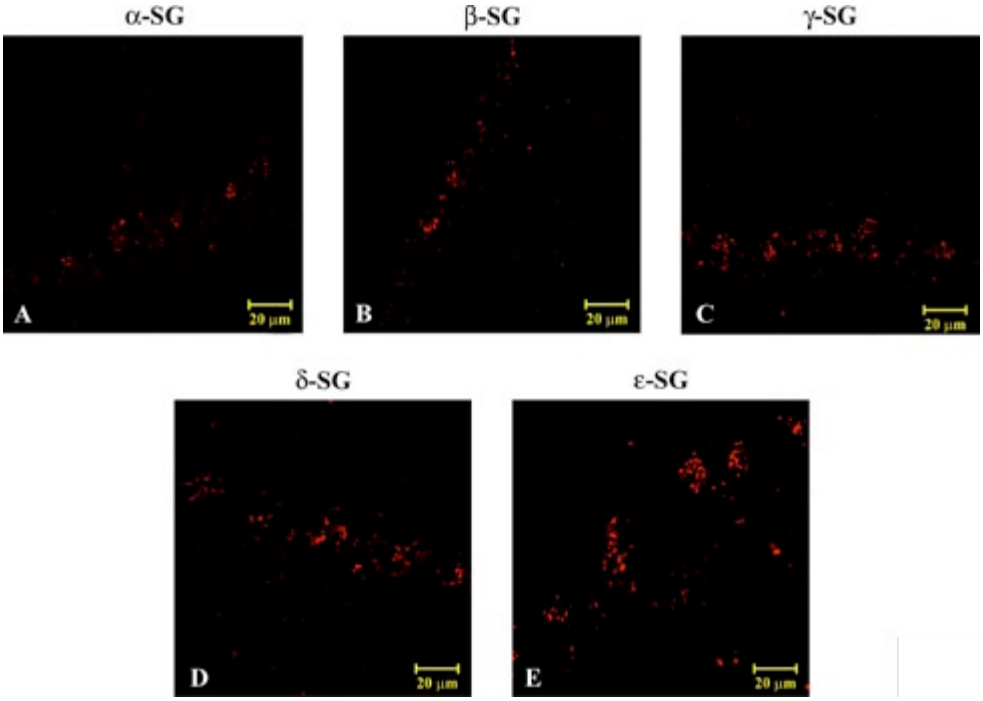

Figure 4 – Confocal image of rat cerebellar cortex labelled with anti-α- (A), β- (B), γ- (C), δ- (D), and ε-sarcoglycans (E) (red channel) antibodies. Red fluorescence is distributed in a single cell layer.

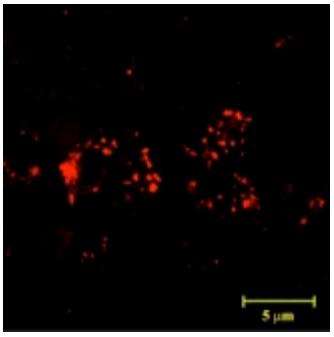

Figure 5 – Typical "spot-like" staining pattern of γ-sarcoglycans (red channel) around the body of the cells, with spots about of 0.5-2 μ

that of the cerebral cortex (Fig. 5); also all other sarcoglycan showed a similar staining pattern (data not shown).

The double localization reaction performed using antibodies against ε-sarcoglycan and SMI-32 (red and green channel respectively in Fig. 6A), and with antibodies against δ-sarcoglycan and GFAP (red and green channel respectively in

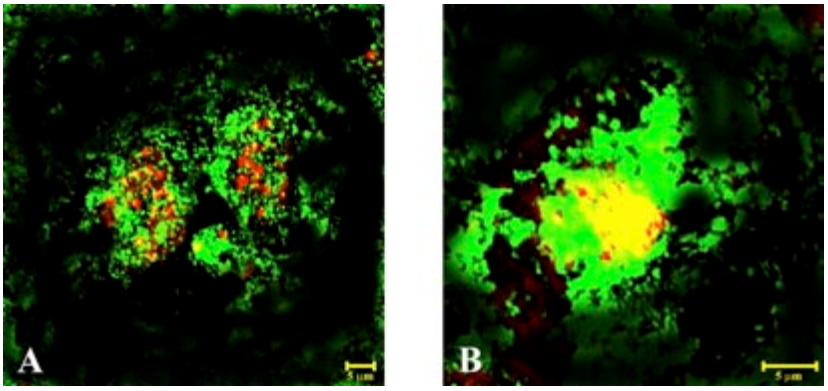

Figure 6 – Confocal image of rat cerebellar cortex double-labelled with anti-ε-sarcoglycan (red channel) and anti-SMI-32 (green channel) antibodies; the fluorescence appears as spot-like (A). Double-labelling with anti-δ-sarcoglycan (red channel) and anti-GFAP (green channel) antibodies; the fluorescence is distributed in the central portion of the cell (B).

Fig. 6B), showed that the sarcoglycans are present both in cerebellar neurons and glial cells. SMI-32 and GFAP appeared also colocalized with all other sarcoglycans (data not shown).

Sarcoglycan positivity in neurons of the cerebellar cortex was present mainly in an area likely corresponding to Purkinje cell layer (Fig. 4), while sarcoglycan positivity in neuronal cells of other regions of cerebellar cortex, like granular and molecular layers, was low or absent.

Discussion

Sarcoglycans are a group of proteins creating a connection between extracellular matrix and cytoskeleton. As well known, in skeletal muscle, this complex stabilizes the sarcolemma of myofibres protecting them from any possible damage provoked by continuing cycles of contraction and relaxation (Ervasti et al., 1990; Yoshida and Ozawa, 1990). Therefore, these proteins have been mainly studied on skeletal, cardiac and smooth muscle fibres (Anastasi et al., 2004), but a few data are available about these proteins in non-muscular tissues. It has been demonstrated that α- and γ-sarcoglycans are specific of skeletal and cardiac muscle, whereas other sarcoglycans are more widely distributed (Roberds et al.,1993; Noguchi et al.,1995; Ettinger et al.,1997; McNally et al.,1998). Moreover, a mutation of ε-sarcoglycan is responsible of Myoclonus Dystonia Syndrome (Zimprich et al.2001), suggesting a key role of this protein in Central Nervous System (CNS); ζ-sarcoglycan was demonstrated to be also highly expressed in brain (Shiga et al., 2006), but there are no data about other sarcoglycans in CNS. It was instead described a complex made up of ε-, β-, δ-, and ζ-sarcoglycans in Schwann cells of peripheral nerve (Cai H. et al., 2007). The data of the present study have shown that in rat cerebral cortex: a) immunofluorescence for all tested sarcoglycans is detectable in all observed regions; b) sarcogly-

cans are present both in neurons and glial cells; c) the staining pattern for all sarcoglycans is "spot-like" around the soma of neurons while in glial cells the labelling is found in the central portion of the cells. In opposition to a report which considered only ε- and ζ-sarcoglycans to be present in brain (Waite et al., 2009), our results show that in rat brain α-, β-, γ-, and δ-sarcoglycans are present too, independently of the species, since we have already demonstrated the expression of α-, β-, γ-, δ- and ε-sarcoglycans in human intra-surgical brain biopsies by immunofluorescence techniques (unpublished data). On this basis we can hypothesize that in brain an hexameric SGC-like exists, similar to pentameric/hexameric SGC described in muscular tissue (Anastasi et al., 2007), but plays a different role if compared with muscle fibres. Sarcoglycans, in the cerebral cortex, appears as intermittent spots about of 0.5-2 μm , mainly around the soma of the neurons. These results suggest that sarcoglycans may play a key role in cellular signalling, regulating membrane synaptic receptors assembly. In particular, the predominant presence of spots around the soma indicate that sarcoglycans may regulate membrane post-synaptic receptors assembly mainly in axosomatic synapses. Moreover it was demonstrated that ε-sarcoglycan is present in many brain regions and that it is associated with dopaminergic neurons (Nishiyama et al., 2004; Chan et al., 2005); preliminary neurochemical analysis of ε-sarcoglycan-deficient mice have shown increased levels of dopamine and its metabolites within the striatum (Yokoi et al., 2006). Thus, ε-sarcoglycan may be involved in dopaminergic neurotransmission (Waite et al., 2009). Besides, it was hypothesized that Dystrophin and the Dystrophin-Glycoprotein Complex (DGC) is involved in modulating synapse function at a subset of GABAergic synapses in the hippocampus and cerebellum (Levi et al., 2002; Kush et al., 2008). On this basis we can speculate that the presence of sarcoglycans around the soma of neurons may marks a specific subset of postsynaptic receptors. Sarcoglycans have been previously studied in Schwann cells of peripheral nervous system were it was demonstrated that they are involved in myelin stabilization (Cai et al., 2007); moreover, it was demonstrated a role of dystrophin-glycoprotein complex of glial cells in the maintenance of the brain-blood-barrier (Haenggi and Fritschy, 2006). Therefore, we can hypothesize that in the glial cells of cerebral cortex these protein may play a role as membrane receptors involved in myelin stabilization and in the maintenance of the brain-blood-barrier. In rat cerebellar cortex: a) all tested sarcoglycans are present; b) sarcoglycans present a spot-like staining pattern mainly around the soma of neurons; c) the sarcoglycan positivity in neurons is detectable mainly in an area which is likely to correspond to Purkinje cell layer, with a low or absent positivity in neurons present in molecular and granular layers; d) sarcoglycans are also expressed in glial cells where their labelling is distributed in a central portion of the cell. Chan et al. (2005) had demonstrated the presence of only ε-sarcoglycan in mouse brain. Our results show that all sarcoglycans are present also in cerebellum cortex of the rat. Sarcoglycan positivity is detectable mainly in a type of neurons, the Purkinje cells. In fact, our results show a low or absent sarcoglycans fluorescence in granular and molecular layers. Moreover, the presence of a sarcoglycans "spot like" staining pattern around the soma of the cells suggest that, also in cerebellum cortex, sarcoglycans might be associated with specific postsynaptic receptors of Purkinje cells. Therefore sarcoglycans may play an important role in Purkinje cell layer, independent of the species. In glial cells of cerebellum cortex sarcoglycans may play the same role which we have hypothesized for them in cerebral

cortex glial cells.

In conclusion, our results shows that α-, β-, γ-, δ- and ε-sarcoglycans are expressed in rat cerebral and cerebellar cortex, where we suppose that they are involved in specific synaptic neurotransmission. These preliminary results could open a new research line concerning the study of sarcoglycans in non-muscular tissues, demonstrating again that these proteins are not muscle-specific; moreover, the information about the presence of sarcoglycans in cerebral and cerebellar cortex, provided by this study, might give birth to further researches. It is interesting to investigate other parts of normal and pathological brain to define a possible involvement of these proteins in modulating synaptic neurotransmission.

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