CORRELATIVE MICROSCOPY OF CEREBELLAR STELLATE NEURONS. A REVIEW

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ABSTRACT
The stellate neurons of cerebellar cortex molecular layer are easily identified at light microscopy, scanning and transmission electron microscopy levels since they are the only short axon nerve cells existing in the middle and outer thirds of cerebellar cortex molecular layer. Some internal details of fractured stellate neurons, such as GERL complex, endoplasmic reticulum and nuclear chromatin have been three-dimensionally viewed by scanning electron microscopy and the freeze-fracture method, taking advantage of the washing out of cytoplasmic soluble proteins from the fracture face induced by the freeze-fracture process. Freeze-etching replica technique for transmission electron microscopy shows the distribution of nuclear pores and the three-dimensional relief of endoplasmic reticulum and cell organelles. The stellate cell axon appears as a unique process directed toward Purkinje secondary and tertiary dendritic branches. The contoured stellate dendrites exhibit a beaded aspect and frequent bifurcations. Parallel and climbing fibers, and basket cell axons establish axospinodendritic and axosomatic contacts with stellate neurons and their dendritic processes. Axodendritic connections between stellate cells also are distinguished. Stellate neurons exhibit immunopositive reactions for Synapsin-I, PSD-95, GluR1, CaMKII and N-cadherin.

Keywords: Stellate neuron, cerebellum, correlative microscopy, immunohistochemistry.

MICROSCOPÍA CORRELATIVE DE LAS NEURONAS ESTRELLADAS CEREBELOSAS

RESUMEN
Las neuronas estrelladas de la capa molecular del cerebelo son facilmente identificables al microscopio óptico, electrónico y de barrido por ser las únicas neuronas de axón corto existentes en el tercio medio y externo de la capa molecular de la corteza cerebelosa. Algunos detalles internos del citoplasma, tales como el complejo de Golgi, el retículo endoplasmático, y el núcleo se visualizan mediante microscopía elctrónica de barrido y la técnica de criofractura para microscopía de barrido, debido al lavado de las proteínas solubles del citoplasma por la técnica de criofractura. La técnica de crifractura para microscopía electrónica de transmisión mostró la distribución de los poros nucleares y el relieve tridimensional del retículo endoplasmático y los organelos celulares. El axón único de las neuronas estrelladas aparece dirigido hacia las ramificaciones dendríticas secundarias y terciarias de las células de Purkinje. Las dendritas muestran aspecto contorneado, aspecto varicoso y frecuentes bifurcaciones. Las fibras paralelas, trepadores y axones de células de cesta establecen sinapsis axosomáticas espinodendríticas asimétricas con las dendritas de las neuronas estrelladas. Tambien se observan sinapsis axodendríticas entre células estralladas. Las neuronas estrelladas muestran reacciones inmunopositivas para Sinapsina I, PSD-95, GluR1, CamKII y N- caderina.

Palabras claves: Neuronas estrelladas, cerebelo, microscopía correlativa,inmunohistoquímica

INTRODUCTION

Stellate cells, the intrinsic interneurons of the cerebellar molecular layer, were earlier described at light microscopy level by Fusari [1], Ramón y Cajal P. [2], Ponti [3], Ramón y Cajal S. [4], Smirnow [5], Estable [6], Jakob [7], Scheibel and Scheibel [8] and Fox et al. [9]. The transmission electron microscopic (TEM) features were earlier studied by Herndon [10], Fox et al. [9], Lemkey-Johnston and Larramendi [11,12], Castejón [13], Sotelo [14,15], Palkovits et al. [16], and Mugnaini [17]. The most complete description of these neurons has been given by Chan-Palay and Palay [18], and Palay and Chan-Palay [19] by means of camera lucid drawings of Golgi light microscopy preparations and transmission electron microscopy (TEM). Castejón and Castejón [20)], and Castejón [21-23] described the TEM features of stellate neurons of mouse cerebellar cortex. Later, Castejón and Castejón [24] reported the glycosaminoglycan content, freeze-etching features, and three-dimensional morphology of stellate neurons by scanning electron microscopy (SEM). Castejón et

al. [25], using SEM and TEM, described the axodendritic connections between granule cell axons or parallel fibers and stellate cell dendrites. Obata et al. [26] firstly described putative GABA-releasing terminals from basket/stellate and Golgi cells immunostained with glutamate decarboxylase-67 antibody. Benagiano et al. [27] demonstrated GABA immunoreactivity in the cell bodies of stellate neurons of human cerebellar cortex by light and electron microscopy. Biagotti et al. [28] found by means of electron microscopy analysis that the basket and stellate cells, as well as the Golgi cells, have a remarkable glucose-6-phosphatase deshydrogenase (G6PD) activity. Corticotropin-releasing factor and urocortin espression in stellate neurons were found by Swinny et al. [29]. Fritschy et al. [30] found GABAergic axodendritic synapses of stellate neurons on Purkinje cells. More recently, Astori et al. [31] have demonstrated GABA release from stellate neurons onto Purkinje cells. Castejón [32] recently described Synapsin-I, PSD-95, GluR1, N-cadherin and Ca $^{2+}$/Calmodulin-dependent Protein Kinase II Alpha immunopositive reactions of stellate neurons.

The present review describes the light and Golgi light microscopy, SEM and TEM features of stellate neurons, and positive immunohistochemical activity of Synapsin-I, PSD-95, GluR1, Calcium/calmodulin-dependent protein kinase II (CaMKII) of stellate neurons, and N-cadherin, and their synapses with granule cell axons, climbing fibers, and from axonal terminals of neighboring stellate neurons.

LIGHT MICROSCOPY

Plastic semithin sections of mouse cerebellar cortex stained with toluidine blue show the triple layered structure of cerebellar cortex formed by granule cell, Purkinje cell and molecular layers. The distribution of stellate neurons can be appreciated in the middle and outer third molecular layer [13]. (Fig.1).

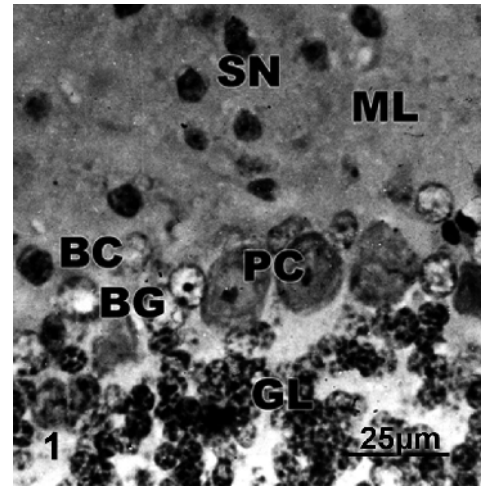

Fig. 1. Mouse cerebellar cortex showing the stellate neuron (SN) distribution in the molecular layer. Basket cells (BC), Bergmann glial cells (BG), Purkinje cells (PC), and the granule cell groups (GC) also are distinguished. (Castejón, 1968).

Golgi light microscopy

Close examination of the molecular layer with Golgi light microscopy technique shows the topographic relationship of stellate neurons with the secondary and tertiary Purkinje dendritic ramifications. The stellate cell axons directed to the Purkinje dendritic processes extend in the middle and outer thirds of molecular layer (Fig. 2).

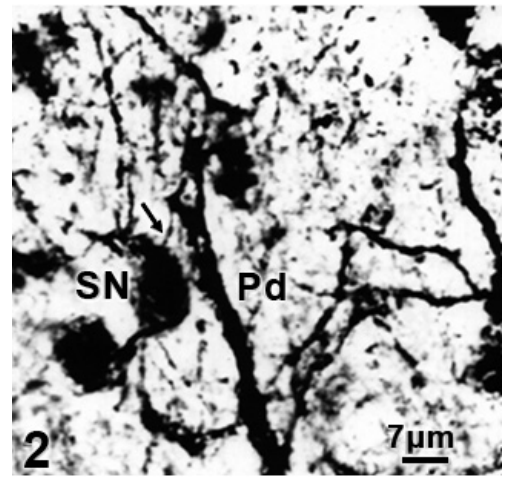

Fig. 2. Mouse cerebellar cortex. Golgi stained thick paraffin section showing a stellate neuron (SN) sending its axonal process (arrow) toward the Purkinje tertiary spiny dendrites (PD). Some stellate neuron dendrites also are seen spreading in the molecular layer (Castejón, 1968).

In addition, Paula-Barboza et al. [33] described by means of the combined Golgi light microscopy and

Golgi-ultrastructural method the stellate cell axonal descending branches forming a pericellular basket around Purkinje cell soma, and contributing to the pinceaux surrounding the Purkinje axonal initial segment, where they establish septate-like junctions.

CONVENTIONAL SCANNING ELECTRON MICROSCOPY

Fish and human cerebellar cortex specimens conventionally processed for conventional SEM, and ethanol-cryofracturing tecnique [34-38] show the superficial short-axon stellate neurons with round, elliptical or fusiform somata in a parasaggital fracture of the outer third molecular layer. These superficial stellate cells are easy to recognize, since they are the only neurons in the upper molecular layer, and appear surrounded by bundles of passing granule cell axons or parallel fibers [39]. (Fig.3).

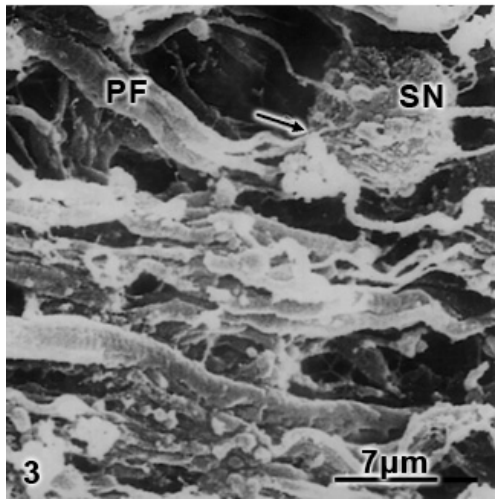

Fig. 3. Teleost fish cerebellar cortex. Scanning electron micrograph showing the stellate neuron (SN) in the outer third molecular layer surrounded by bundles of parallel fibers (PF). The arrow indicates the parallel fibers approaching to the stellate cell soma (Castejón, 1988).

Three to five beaded short and ramified dendrites radiate from the cell body toward the neighboring Purkinje dendrites or other stellate cells [36-38]. The axon originates by way of a typical triangular shaped axon hillock and, after a short initial segment bifurcates into tenuous varicose collaterals. The short axonal process directed to the Purkinje cell dendrites, and the convoluted and cryodissected dendritic processes can be appreciated in SEM human cerebellar cortex prepared by means of ethano-cryofracturing tecnique of Humphreys et al. [35]. (Figs. 4 and 5).

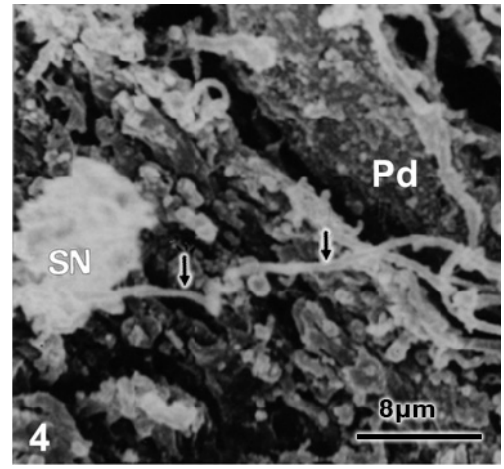

Fig. 4. Scanning electron microscopy of human cerebellar cortex showing a stellate neuron (SN), and its axonal processes (arrows) directed toward the Purkinje dendrite (Pd). Gold-paladium coating. (Castejón and Castejón, 1987).

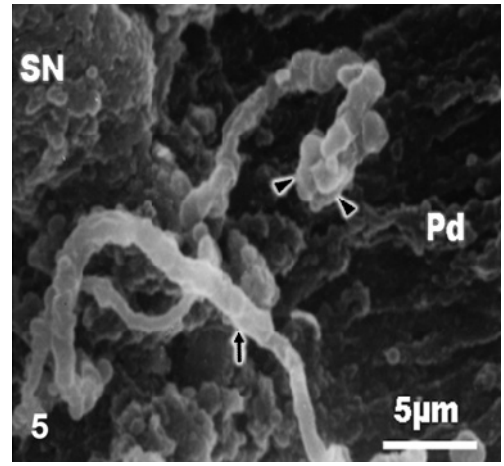

Fig. 5. Human cerebellar cortex. Conventional SEM and ethanol-criofracturing technique. Outer surface of a stellate neuron (SN) showing the dendritic processes (arrow) ending on dendritic twigs (arrowhead). (Castejón and Castejón, 1987).

SEM and SEM freeze-fracture technique

By means of the freeze-fracture technique for SEM [25,35-40], the stellate neurons are fractured through the equatorial plane showing at low magnification the condensed pattern of nuclear heterochromatin, and the three-dimensional image of the GERL complex [41,42], formed by the Golgi cisternae and their sacs,

endoplasmic reticulum canaliculi, and lysosomes. The dendrites exhibit also the outer surface of endoplasmic reticulum. The inner cytoplasmic details have been visualized taking advantage of washing out of cytosol soluble proteins induced by the SEM freeze-fracture process [43,44]. (Fig.6).

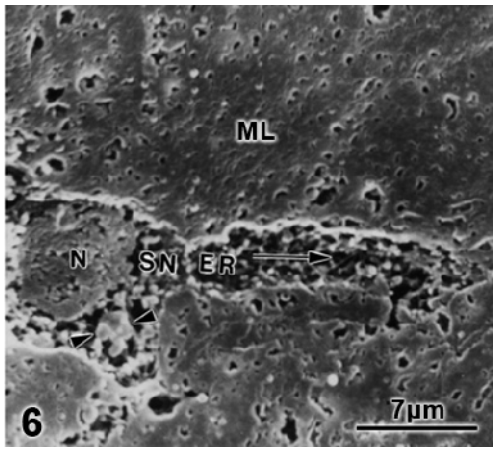

Fig. 6. Teleost fish cerebellar molecular layer (ML) showing the fractured stellate neuron (SN). SEM freeze-fracture method. The nucleus (N), the GERL complex (arrowheads), and the endoplasmic reticulum (ER) are observed extending from the cell body to the dendritic process (arrow). (Castejón, 1988).

TRANSMISSION ELECTRON MICROSCOPY

Stellate cell soma

The fine structure of stellate neurons shows the general features of a microneuron characterized by a scarce band of perinuclear cytoplasm containing rough endoplasmic reticulum canilículi, free ribosomes and polysomes, bundles of microtubules, motochondria, Golgi complex, lysosomes, and coated vesicles [24]. (Fig.7).

In addition, Ruela et al. [45] earlier described cilia in stellate neurons of rat cerebellum. Castejón and Castejón [24] demonstrated by means of light and electron microscopy histochemistry the presence of a homogenous alcianophilic substance within the stellate neuron cytoplasm characterized as a glycosaminoglycan sensitive to hyaluronidase treatment, mainly hyaluronic acid.

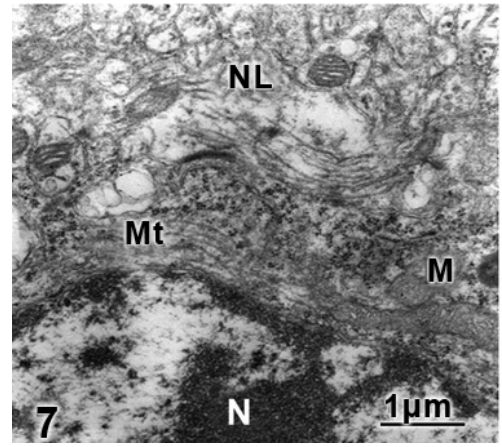

Fig.7. Mouse cerebellar cortex. Transmission electron micrograph of stellate neuron showing the nucleus (N), the cytoplasm containing scarce profiles of endoplasmic reticulum, numerous free ribosomes, bundles of microtubules (Mt), and mitochondria (M). The neighboring neuropil (NL) of molecular layer also is noted. (Castejón and Castejón, 1987).

Monteiro [46] established by means of a morphometric analysis at TEM level statistically significant differences in data concerning perikaryon volume, perikaryon surface and intracellular organelle composition between basket and stellate cells, and postulated that each class of interneuron should be designated with a specific name.

A complex neuropil formed by the Purkinje-parallel and climbing fiber spine synapses, surrounded by the Bergmann glial cell cytoplasm is observed adjacent to the stellate neurons.

Stellate neuron synapses

According to earlier TEM studies, climbing and parallel fibers, and basket cell axons establish axosomatic and axodendritic contacts with stellate cells [11,19,24,39,47,48]. Small synaptic buttons of climbing fibers, "en passant" parallel fibers, basket cell and Lugaro cell axons, or axonic terminals of neighboring stellate neurons are observed attached to the somatic neuronal surface. Basket cell endings exhibit ellipsoidal and flattened synaptic vesicles. On the contrary, large synaptic endings of climbing fiber characterized by the presence of spheroid synaptic vesicles are observed making axodendritic junctions [49,50]. (Figs.8 and 9).

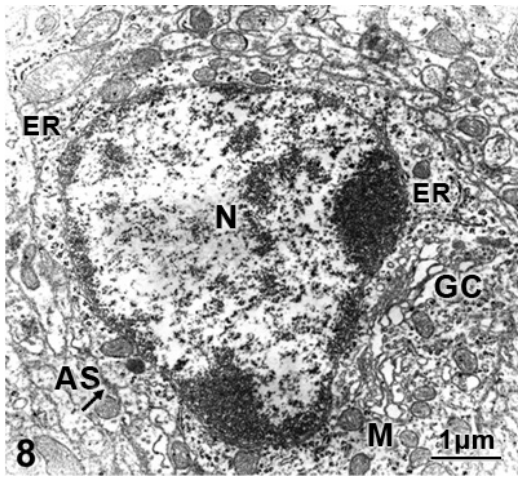

Fig. 8. Mouse cerebellar molecular layer. TEM of stellate neuron displaying the nucleus (N), the mitochondria (M), Golgi complex (GC), scarce profiles of rough endoplasmic reticulum (ER), and an axosomatic synapse (AS, arrow). (Castejón and Castejón, 1987).

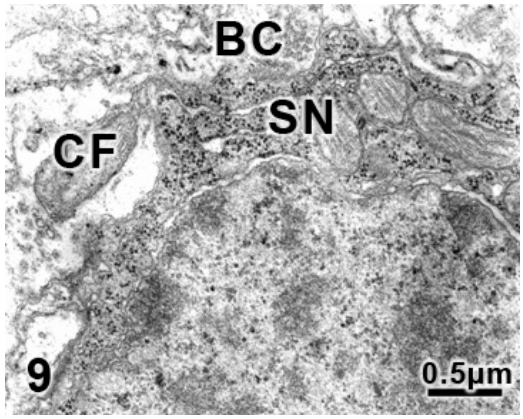

Fig. 9. Mose cerebellar cortex. Stellate neuronal soma (SN) exhibiting axosomatic synapses with a large climbing fiber synaptic ending (CF) characterized by spheroid synaptic vesicles, and with a small basket cell ending (BC) featured by flattened vesicles.

TEM and the freeze-etching technique

With the freeze-etching technique the smooth fractured cytoplasm of stellate cells is observed exhibiting the three-dimensional relief of endoplasmic reticulum and cell organelles embedded in a smooth surface cytosol. Axodendritic contacts of climbing fibers also are found (Fig. 10).

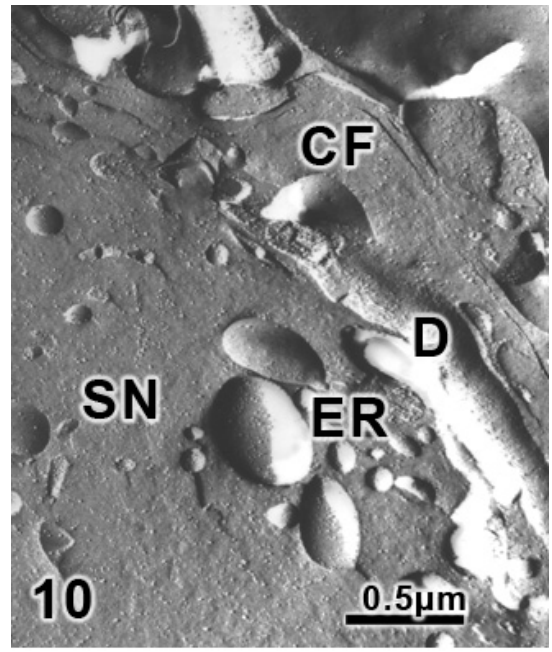

Fig. 10. Mouse cerebellar molecular layer. TEM and freeze-etching technique. Stellate neuron cytoplasm (SN) displaying the endoplasmic reticulum profiles, free ribosomes embedded in the cytoplasm, and a dendritic process (D). A large climbing fiber synaptic ending (CF) appears synaptically apposed to the dendritic process (Castejón et al., 2000).

CONFOCAL LASER SCANNING MICROSCOPY AND IMMUNOHISTOCHEMISTRY

Synapsin-I immunohistochemistry

Slices of rat cerebellar cortex labeled with Synapsin-I show the distribution of small puncta surrounding the stellate neurons and their process corresponding to the distribution of presynaptic endings of climbing fiber and parallel fibers [32,51]. (Fig. 11).

PSD-95 immunohistochemistry

Slices of rat cerebellar cortex labeled with PSD-95 show the immunopositive stellate neurons and their process corresponding to the distribution of postynaptic endings of climbing fiber and parallel fibers [32,51]. (Fig. 12).

PSD-95 is a postsynaptic protein predominantly associated with postsynaptic densities [51].

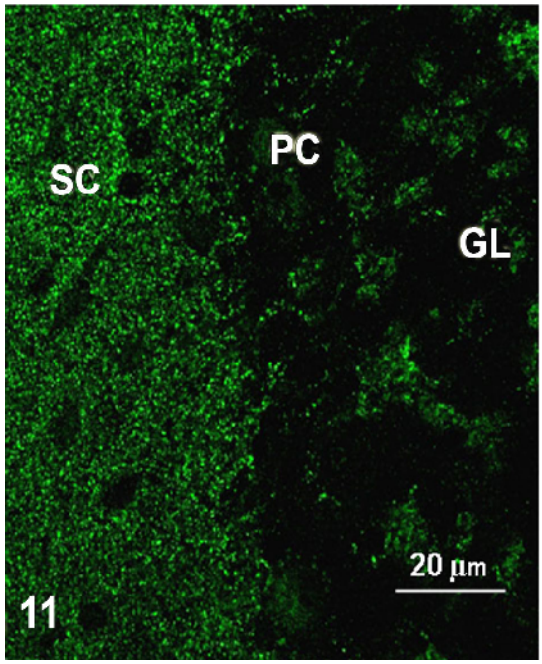

Fig.11. Rat cerebellar cortex. Note the small puncta expressing Synapsin-I positive immunolabeling surrounding the stellate cells (SC) at the molecular layer. The Purkinje cell layer (PC) and granular layer (GL) also are appreciated. (Castejón and Dailey, 2009).

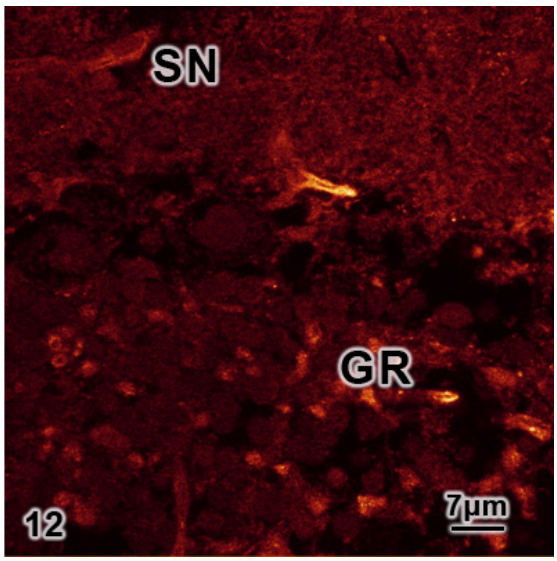

Fig.12. Rat cerebellar cortex immunolabeled with PSD-95 showing at the level of molecular layer the immunopositive stellate neuron (SN) corresponding to their postsynaptic axosomatic and axodendritic sites. Note also the immunopositive postsynaptic sites of granule cell dendritic tips at the glomerular regions (GR) in the granular layer.

GluR1 subunit immunohistochemistry

Rat cerebellar slices labeled with PSD-95 show small red puncta corresponding to GluR1 subunits of AMPA receptors surrounding stellate and basket cells in the rat molecular layer [32,52]. (Fig. 12).

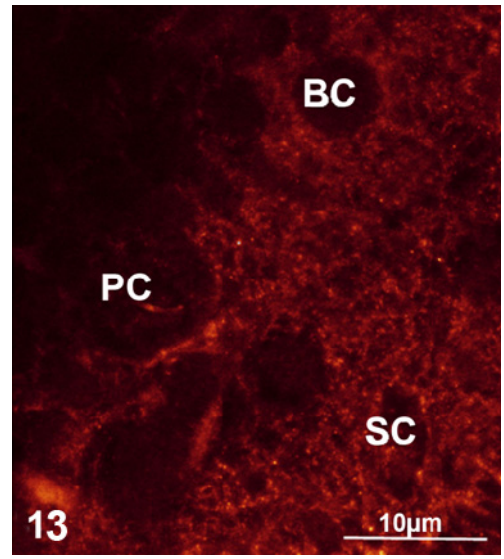

Fig.13. High magnification of rat cerebellar slice double labeled with anti-GluR1 monoclonal antibody. The secondary antibody used was Alexa-488 goat anti-rabbit (GAR) IgG. Note the GluR1 immunoreactivity of stellate cell soma (SC) and processes, basket cell (BC), and Purkinje cell body (PC). (Castejón, 2010).

The distribution of GluR1 subunits relates this postsynaptic AMPA receptor subclass to the excitatory circuits of the cerebellar cortex formed by climbing and parallel fiber synapses upon stellate neurons. Thus far, the expression of another subunit, the GluR2 subunits-containing AMPA receptors has been reported only on cerebellar stellate cells [53,54].

N-cadherin immunohistochemistry

Rat cerebellar slices double labeled with a primary antibody against N-cadherin, and Alexa 488 goat anti mouse (GAM)-antibody [32] show strong punctate immunostaining at the level of soma and processes of stellate neurons (Fig. 14).

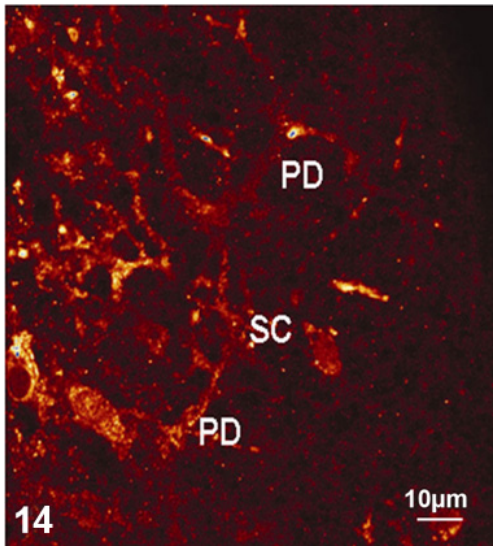

Fig.14. Rat cerebellar slice. N-cadherin immunostaining showing immunopositive reaction of soma and processes of a stellate cell (SC), and Purkinje dendritic ramifications (PD). (Castejón, 2010).

N-cadherin is a membrane glycoprotein mediating strong homophilic adhesion and concentrated at the synaptic junctions and neural circuits, where they exert an active role in synaptic structure, function, plasticity, and in selective interneuronal connections during network function [55- 60],

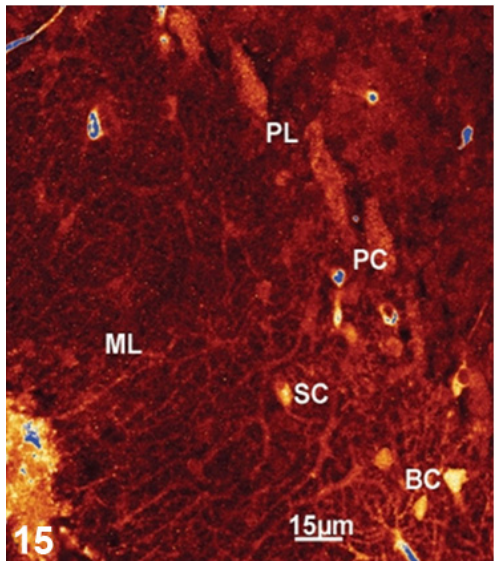

Fig.15. Rat cerebellar slice double labeled with double labeled with a primary antibody against CaMKII alpha, and a secondary antibody the Alexa 488 goat anti mouse (GAM)-antibody. Note the immunopositivity of stellate, basket and Purkinje cells.

Immunohistochemistry of Ca $^{2+}$/Calmodulin-dependent Protein Kinase II Alpha

Stellate cell cytoplasm and processes show strong immunopositive reaction for Ca $^{2+}$/Calmodulin-dependent Protein Kinase II Alpha [32]. (Fig. 15).

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a Ca^{2+}- activated enzyme that is highly abundant in the brain and play a major role in Ca^{2+}-mediated signal transduction. CaMKII constitute a family of multifunctional protein kinase isoforms (alpha, beta, gamma and delta).[61,62]. Lisman et al. [62] have postulated their role in long-term information storage, motor learning and long-term synaptic memory.

CONCLUSIONS

Correlative microscopy of stellate neurons made by light microscopy, transmission electron microscopy and freeze-etching technique, scanning electron microscopy and cryofracture method, as wells as the use of immunohistochemical techniques for confocal laser scanning microscopy have permitted a better and deeper understanding of cerebellar structure and function, mainly regarding the three-dimensional morphology of outer neuronal surface, intramembrane morphology, stellate cell synaptic axospinodendritic and axosomatic contacts with parallel and climbing fibers, and the precise localization of pre- and postsynaptic receptors by means of immunohistochemical techniques for Synapsin-I, PSD-95, and GluR1 subtype of AMPA receptors. Stellate neuron synaptic contacts with Purkinje cell demonstrate the neural correlates of the inhibitory action of stellate cells upon Purkinje cells. The axosomatic and axodendritic presynaptic contacts of climbing and parallel fibers as demonstrated by transmission electron microscopy and Synapsin-I and PSD-95 evinces de excitatory action of these afferent fibers upon stellate neurons. The GluR1 immunopositivity of stellate neurons reveals the presence of glutamatergic neurotransmission at the level of stellate neuron synapses. The CaMKII immunopositivity of stellate neurons suggest their role

in long-term information storage, motor learning and long-term synaptic memory. The N-cadherin positive immunoreaction of stellate nerve cells and their intracortical circuits demonstrate the active role of these cell adhesion molecules in synaptic structure, function, and plasticity.

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