ELECTRON MICROSCOPY AND CONFOCAL LASER SCANNING MICROSCOPY OF LUGARO CELLS OF VERTEBRATE CEREBELLAR CORTEX. A REVIEW.

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ABSTRACT

The present review shows the outer surface morphology of Lugaro cells as seen by scanning electron microscopy. They are easily characterized by their location beneath Purkinje cells. Confocal laser scanning microscopy and immunochemistry technique for calbindin revealed positive immunoreaction for cell body, and axonal and dendritic processes, and calcium binding proteins. Immunopositive reactions for Synapsin-I, Synapsin I and PSD-95, GluR1, CamKII alpha, and N-cadherin are found. Synapsin-I immunopositivity shows the localization of presynaptic terminals. Synapsin-I and PSD-95 shows the precise localization of Lugaro cell pre- and postsynaptic junctions of axosomatic and axodendritic contacts. GluR1 immunopositive reaction indicates that Lugaro cells have functional ionotropic glutamate receptors. CaMKII alpha immunopositivity of Lugaro cell suggest its participation as a molecular switch for long-term information storage, and serving as a molecular basis of long-term synaptic memory. N-cadherin immunopositivity demonstrates the significance of this cell adhesion molecule for Lugaro cell synaptic structure and function.

Keywords: Lugaro cells, cerebellum, microscopy, immunohistochemistry.

MICROSCOPÍA ELECTRÓNICA Y MICROSCOPÍA CONFOCAL DE RAYOS LASER DE LAS CÉLULAS DE LUGARO DE LA CORTEZA CEREBELOSA DE LOS VERTEBRADOS. REVISIÓN

RESUMEN

La presente revisión muestra la morfología de las células de Lugaro vista con el microscopio electrónico de barrido. Ellas se localizan facilmente por su ubicación debajo de las células de Purkinje. La técnica inmunohistoquímica para calbindina muestra el cuerpo celular, las prolongaciones dendríticas y el contenido de proteínas ligadas al calcio. Se observa inmunopositividad para Sinapsina-I, Sinapsina-I y PSD-95, GluR1, CamKII y N-caderina. Sinapsina-I muestra la localización de los terminales presinápticos. Synapsina-I y PSD-95 muestran la localización de las uniones pre- y postsynapticas de los contactos axosomáticos y axodendríticos de las células de Lugaro. GluR1 muestra que las células de Lugaro contienen receptores ionotrópicos de glutamato funcionantes. La inmunopositividad para CamKII alfa demuestra la participación de las células de Lugaro como un switch para almacenamiento de información de largo plazo, sirviendo como la base molecular para la memoria de largo plazo. La inmunopositividad de la N-caderina revela la significación de esta molécula de adhesión celular en la estructura y función de las sinapsis de las células de Lugaro.

Palabras claves: Células de Lugaro, cerebelo, microscopía , inmunohistoquímica.

INTRODUCTION

Cajal [1] carried out the pioneering study of Lugaro cells by means of Golgi technique. According to Cajal, Lugaro cells exhibit an axon directed downwards to the granular layer and reaching the white matter. He observed their horizontally directed dendrites in connection with the basket cell descending axonal collaterals, forming the pinceaux around the axon hillock region of Purkinje cell. The Lugaro cells were later described by Fox [2] using Golgi light microscopy as spindle shape cells transversely oriented in the granular layer and located

immediately beneath the Purkinje cell layer. Fox also traced descending dendrites in synaptic relationship with mossy fiber rosettes at the level of mossy glomerulus, and with the Golgi cell axonal ramifications. According to this author, the axonal collaterals of Lugaro cells contact the basket cell bodies in the lower molecular layer. Sinton [3] earlier published the fine structural observations on Lugaro and Golgi cells.

O "Leary *et al*. [4] made a detailed study of Lugaro cell and their processes in the granular and molecular layer. Braak [5] reported a pigmento-architectonic study of Lugaro cells.

Palay and Chan-Palay [6] reported two kinds of horizontal fusiform cells possessing different axonal pattern and distribution.

Laine and Axelrad [7,8] reported Lugaro cells with axons projected to the molecular and granular layers targeting stellate and basket cells.

Melik-Musyan and Fanardzhyan [9] described two Lugaro cell types with fusiform and triangular cell bodies. According to these Authors, Lugaro cells exhibit also projection areas ranging from the molecular layer to the white substance.

De Camilli *et. al*. [10] reported by means of guanosine 3':5'-phosphate-dependent protein kinase antiserum, a specific immunohistochemical marker for the dense innervation by Purkinje cell recurrent axonic collateral around large interneurons, such as Lugaro and Golgi cells, and around the Purkinje cell pinceaux.

Gruol and Crimi [11] identified Lugaro cells in culture using immuno- histochemical techniques and antibodies to gamma-aminobutyric acid (GABA), parvalbumin, and cyclic guanosine monophosphate-dependent protein kinase. Sahin and Hockfield [12] characterized Lugaro cells with the Lugaro cell-type-specific antibodies: Cat-301 and Cat-304, as a distinct cell type from Golgi cell. According to these Authors, Lugaro cells establish numerous contacts almost with all cortical nervous elements: Purkinje and Golgi cells,

granular and basket neurons, parallel and mossy fibers, and even unipolar brush cells.Using electronmicroscopic gold-toning procedure and post-embedding anti-GABA immune-cytochemistry, they demonstrated that Lugaro cell axon forms multiple symmetrical synaptic connections with basket and stellate cell soma and proximal dendrites. In addition, Lugaro cell partially myelinated axons form a parasagittal plexus that participate in the synaptic contacts of Lugaro cells with Golgi cells, but also there are extend long transverse branches that run parallel to the parallel fibers in the molecular layer.

Negyessy *et. al*. [13] demonstrated by light microscopy (LM) and transmission electron microscopy (TEM), using pre-embedding immunoperoxidase and immunogold techniques, the cellular and subcellular localization of mGluR5 metabotropic glutamate receptor in Golgi, Lugaro cells and in parallel fiber synaptic contacts. Lugaro cells lying under Purkinje cell bodies are stained intensely using histochemical demonstration of NADPH-diaphorase (NADPH-d) [14].

Dendro-somatic and somato-somatic contacts of Lugaro cells, and dendro-dendritic contacts between Lugaro and Golgi cells also were reported by these Authors. Puschina and Varaskin [15] demonstrated argyrophilic and nitric oxidergic Lugaro cells.

Geurts *et. al*. [16] reported that rat-303/calretinin double-labeled cells located just underneath the Purkinje cell layer represented Lugaro cells. Moreover, double anti-calretinin and anti-calbindin immunolabellings show that Lugaro cells as well as some globular somata dispersed in the granular layer are both calretinin-positive, and in close apposition with numerous calbindin-positive varicosities of Purkinje cell axon recurrent collaterals. These latter are known from previous ultrastructural studies to be pre-synaptic to Lugaro cells [16].

The common granular layer location and calretinin labelling, the striking similarity in axonal projection pattern, and the important common recurrent

afferentation by Purkinje cell axons strongly argue in favor of the classification of these globular interneurons as a subgroup of a widened Lugaro cell type [17].

Vig *et. al*. [18] observed Lugaro-like cells in the white matter and internal granular layer of the cerebellum of young cats. Bastianelli [19] showed that Lugaro and unipolar brush cells present an opposite immunoreactivity profile, most of them being calretinin positive while lacking calbindin-D28k and parvalbumin. Castejón [20] described the outer surface morphology of Lugaro cells using scanning electron microscopy.

Flace *et. al*. [21] included Lugaro cells into the 'Non-traditional' large neurons of the granular layer of the cerebellar cortex examined by means of immunocytochemistry for glutamic acid decarboxylase (GAD). These morphological data further pointed out the possible functional roles for GABA as a neurotransmitter/neuromodulator in the intrinsic, associative and projective circuits of the cerebellar cortex.

Rodrigo *et. al*. [22] described intense immunostaining for neuronal nitric oxide synthase of Lugaro cells, including unipolar brush cells, and Golgi neurons in sheep cerebellum, which are not immunoreactive in rodents.

Sotnikov [23] postulated that Lugaro cells in the cerebellum and various synaptically NO-positive neurons in the cerebral cortex form part of sensory innervation of the brain. Crook *et. al*. [24] found that interneurons in the granular layer are glycine positive at a density 120 cells/linear mm. Their morphology indicates that they include Lugaro cell and Golgi cell types with the majority containing both glycine and GABA or glutamic acid decarboxylase.

Simat *et. al*. [25] has demonstrated that Lugaro and globular cells are glycinergic/GABAergic and lack mGluR2 and neurogranin. Nunzi and Mugnaini [26] found expression of secretogranin II, chromogranin A and chromogranin B in Lugaro cells. Castejón [27] described immunopositive reactions for Synapsin-I,

Synapsin I and PSD-95,GluR1, CamKII alpha, and N-cadherin. Synapsin-I immunopositivity demonstrated the synaptic relationship with extrinsic afferent (mossy and climbing fibers) and intrinsic afferents to Lugaro cells. N-cadherin immunopositivity was correlated with somato-somatic and somato-dendritic junctions between Lugaro cells and their synaptic connections with other cerebellar neurons.

Hirono *et. al*. [28] distinguished in the upper granular layer, three types of smaller-sized inhibitory interneurons identified on the basis of morphological criteria: small Golgi cells, small fusiform Lugaro cells, and globular cells. According to these Authors the size of Lugaro cells are 119 μm^2, and exhibit three primary processes with an initial branch points soma about 18.4 μm. The anatomical study shows that the axon collaterals of Purkinje cells make synaptic contacts with Lugaro cells and globular cells in the adult cerebellum.

Outer Surface Morphology of Lugaro Cells as Seen by Freeze Fracture Scanning Electron Microscopy

In teleost fish cerebellar cortex examined by conventional SEM, Lugaro cells appear as ovoid cells located underneath Purkinje cells, and showing an ascending axon directed toward the molecular layer and horizontal dendrites remaining in the granular layer [20] (Fig.1).

Transmission Electron Microscopy

Lugaro cells usually appear as small interneuron located beneath the Purkinje cell. They exhibit a clear cytoplasm containing scarce development of rough and smooth endoplasmic reticulum, mitochondria, lysosomes, and a round nucleus (Fig.2).
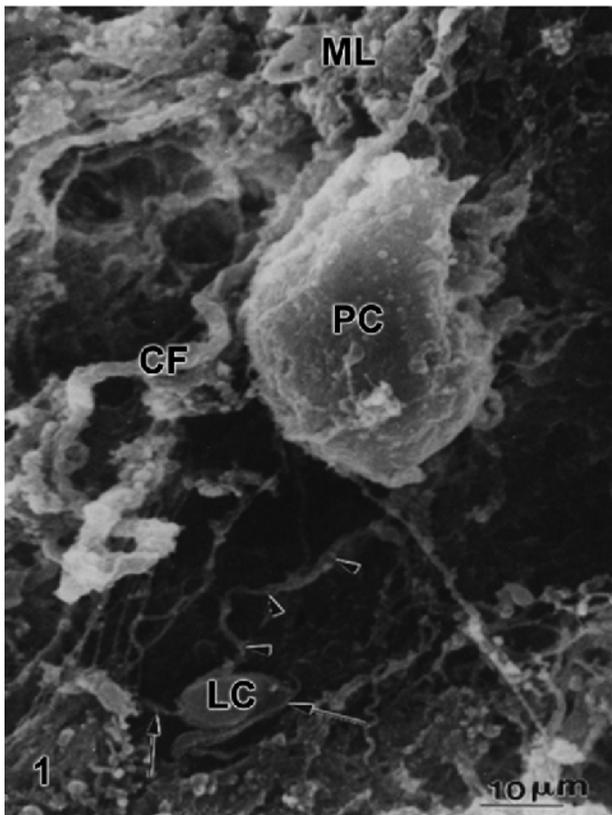
Fig.1. Cryofractured teleost fish cerebellar cortex. Freeze-fracture SEM method. Lugaro cell (LC) showing an ovoid cell body, and an ascending axon (arrowheads) directed toward the molecular layer, and horizontal dendrites (arrows) remaining in the granular layer. A climbing fiber (CF) is also observed approaching to the Purkinje cell soma (PC), and ascending to the molecular layer (ML). Gold-palladium coating.

Fig. 2. Mouse cerebellar cortex. Transmission electron micrograph of a Lugaro cell nucleos (LC) beneath Purkinje cell (PC). Note the presence of its clear cytoplasm, (asterisk) mitochondria (M), and a lysosome (L). Cross sections of a myelinated axon (Ma) also is distinguished.
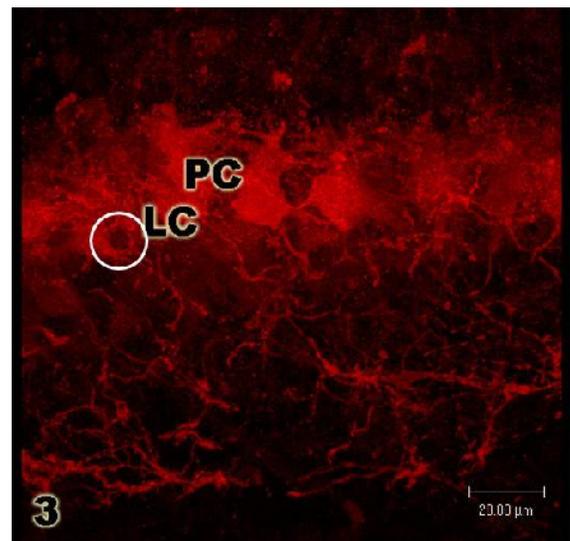
Fig.3. Rat cerebellar slice labeled with calbindin showing positive immunostaining of calcium binding proteins of Lugaro cell (LC) soma and processes. Note its location beneath Purkinje cell (PC) layer.

Confocal Laser Scanning Microscopy (CLSM)

Calbindin immunohistochemistry

Slices of rat cerebella cortex labelled with calbindin and examined with CLSM show intense labelling of Lugaro cell cytoplasm (Fig. 3), the axonal process directed toward the granular layer, the horizontal dendrites remaining in the granular layer, and the ascending dendritic processes going to the Purkinje cell and molecular layers (Fig. 3).

Calcium plays a fundamental role in the cell as second messenger and is principally regulated by calcium-binding proteins. Our findings are different from Bastianelli studies [19] who report calbindin negative Lugaro cells.

According to this Author the function of these proteins is not fully understood, although strong evidence supports a prominent role in physiological settings with altered calcium concentrations. These proteins regulate and are regulated by intracellular calcium level. For example,

they may directly or indirectly enable sensitization or desensitization of calcium channels, and may further block calcium entry into the cells, like the calcium-sensor proteins, that have been shown to be potent and specific modulators of ion channels, which may allow for feedback control of current function and hence signaling.

Synapsin-I-Immunohistochemistry

Synapsin-I, one of the major synaptic phosphoproteins, associates with synaptic vesicles, and regulates neurotransmitter release. In mature neurons, it is concentrated primarily in presynaptic nerve terminals where it appears to mediate interactions of synaptic vesicles with actin filaments and microtubules [29]. Strong, punctate immunoreactivity with synapsin-I antibodies is a reliable marker of presynaptic structures, and therefore has been used widely for immunohistochemical analysis of synapse formation and distribution [30]. Rat cerebellar slices labeled with Synapsin I show the distribution of presynaptic endings surrounding the Lugaro cell soma (Fig.4), presumably corresponding to mossy and climbing fiber collaterals, and Purkinje cell recurrent collateral axons.
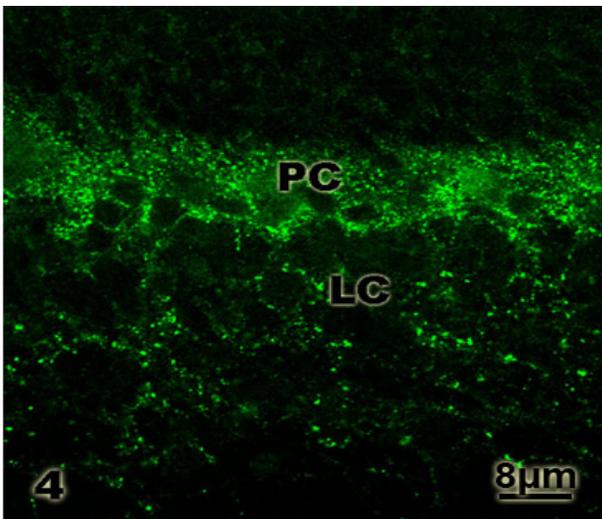
Fig. 4. Slice of rat cerebellar cortex labeled with Synapsin-I showing positive immunostaining expressed by tiny green dots surrounding Lugaro cells (LC) soma and processes corresponding to presynaptic terminals impinging upon Lugaro cell.

Synapsin-I and PSD-95 immunohistochemistry

Slices of cerebellar cortex labeled with primary and secondary antibodies against Synapsin-I and PSD-95. show small green puncta surrounding the Lugaro cell body corresponding to the presynaptic fibers making axosomatic contacts, and large red hotspots deposited at subcellular and cellular body localization, corresponding to the postsynaptic sites of afferent extrinsic and intrinsic fibers (Fig.5).
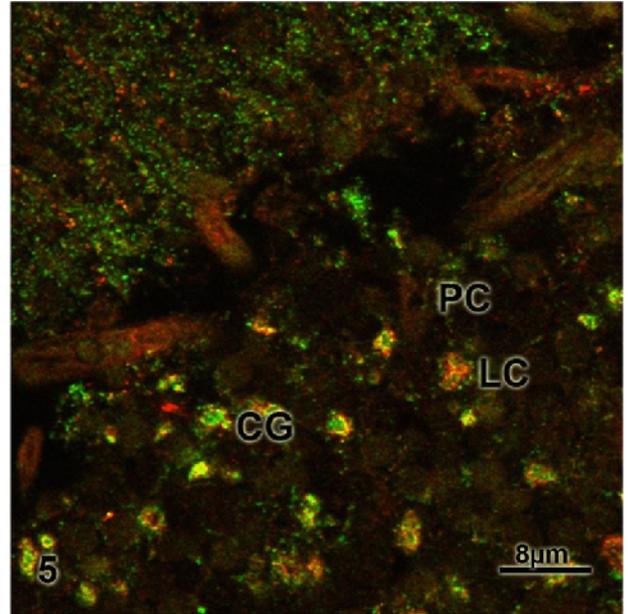
Fig. 5. Rat cerebellar cortex labeled with anti-synapsin-I rabbit IgG polyclonal antibody and anti-PSD-95 (mouse) primary antibody, and a primary antibody against calbindin followed by Cy5 as a secondary antibody. The secondary antibodies were Alexa-488 goat anti-rabbit (GAR) IgG and Alexa-568 goat-anti-mouse IgG. Positive Synapsin-I immunoreactions appears as green tini dots surrounding Lugaro cells. PSD-95 immunopositivity appears as red hotspots within the Lugaro cell peripheral cytoplasm.

Glur1 Immunohistochemistry of Ampa Receptors

GluR1 is one of the several subunits of the subclass quisqualate (QA) receptors couplex to cationic ionic channel, also termed AMPA receptors. Our observations of GluR1 subunit immunohistochemistry reveals the presence of this subclass of AMPA receptors surrounding Lugaro cells and other cerebellar inhibitory cerebellar neurons (Purkinje, basket and stellate cells) [31]. Positive

immunofluorescence staining is observed at the cell soma and processes of Lugaro cell (Fig. 6).
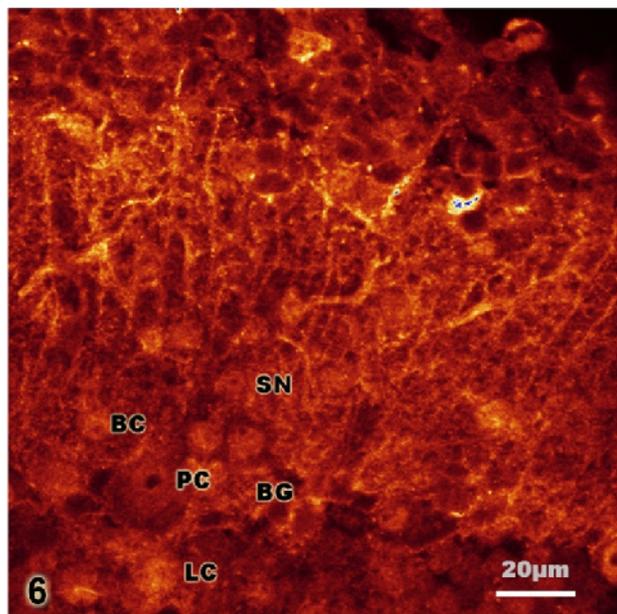
Fig. 6. Rat cerebellar slice labeled with anti-GluR1 monoclonal antibody. The secondary antibody was Alexa-488 goat anti-rabbit (GAR) IgG. Lugaro cells (LC) and processes show positive immunostaining. Purkinje cell (PC), basket cell (BC), Bergmann glia (BG), and stellate neuron (SN) also are seen.

These findings mean that Lugaro cells have functional GluR1 ionotropic glutamate receptors that regulate calcium levels [27].

CaMKII alpha immunohistochemistry

Confocal laser scanning microscopy observations, by means of stack of optodigital sections spanning 28 μm in the granular layer, show punctate CaMKII alpha immunoreactivity at the level of Lugaro cell soma and processes [27] (Fig.5).

The CaMKII has an apparent cytosolic localization as earlier revealed by subcellular fractionation studies of rat brain [32]. CaMKII beta is present in cell bodies and dendrites in mature hippocampal neurons [33]. Previous studies by means of biochemical methods, electron microscopy and confocal laser scanning microscopy have

localized the CaMKII at the level of postsynaptic density [34,35], being central to the regulation of glutamatergic synapses. According to Lisman *et. al*. [35], this localization indicates that a binding pattern for CaMKII is the NMDA receptor within the postsynaptic density. The AMPA receptor subunit GluR1 is also phosphorylated by CaMKII enhancing channel function. The CaMKII has been postulated by Lisman *et. al.* [35] as a molecular switch for long-term information storage, and serving as a molecular basis of long-term synaptic memory. a form of synaptic plasticity associated with learning and memory [35-37] The expression of CaMKII is developmentally regulated [38-39].
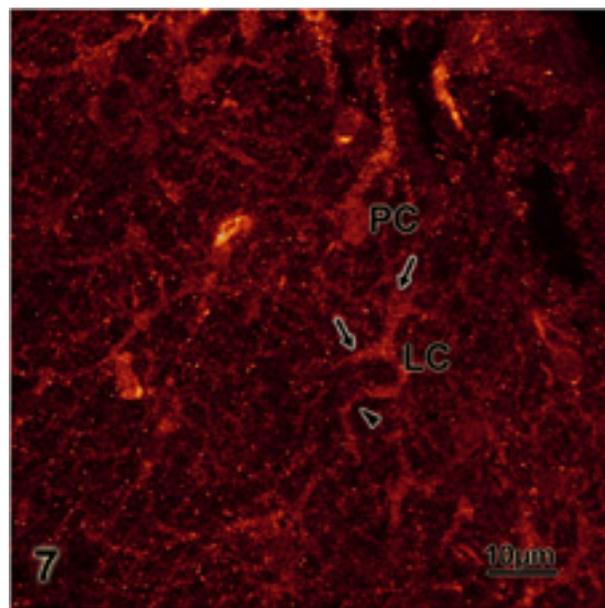
Fig. 7. Rat cerebellar slice labeled with a primary antibody against CaMKII alpha, and Alexa 488 goat anti mouse as a secondary antibody. Small puncta are observed surrounding Lugaro cell soma (LC), descending axon (arrowhead), and the horizontal and ascending dendrites (arrows). (PC) labels a clear Purkinje cell.

N- Cadherin Immunohistochemistry

N-cadherin, a membrane of the Ca^{2+}-dependent cell adhesion molecule family, play essential and specific roles in morphogenesis and histogenesis, as well as in

the transduction of long-range growth and differentiation signals of nerve and muscle cells [40.41].

Rat cerebellar slices exhibit strong N-cadherin immunoreactivity at the level of Lugaro cells (Fig.6), which is apparently related with the dendro-somatic and somato-somato contacts between Lugaro cells, and dendro-dendritic contacts between Lugaro and Golgi cells [27].
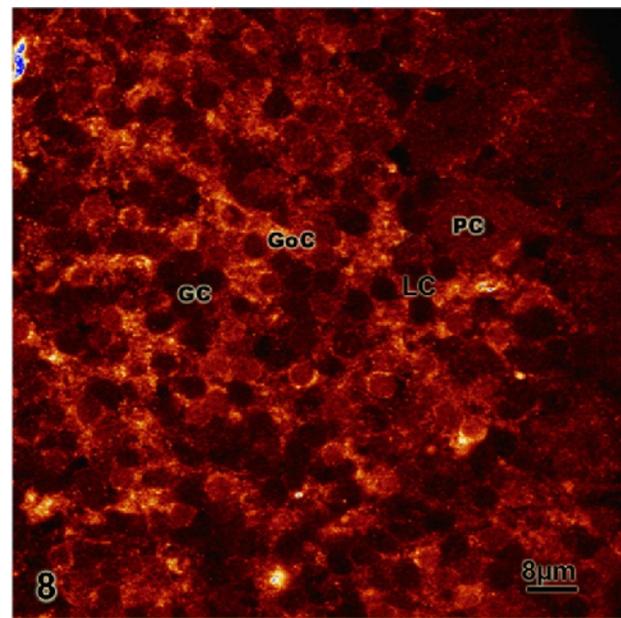
Fig. 8. Rat cerebellar slice Slices were double labeled with a primary antibody against N-cadherin and Alexa 488 goat anti mouse (GAM) secondary antibody showing positive immunochemistry of Lugaro cells (LC) located beneath Purkinje cell (PC). Also note the positive immunochemistry of Golgi cell (GoC), and the unstained granule cell groups (GC).

N-cadherin is a membrane glycoprotein mediating strong homophilic adhesion and also 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 [40, 41]. Another member of the cell adhesion molecule cadherin family, the M-Cadherin was earlier found by Rose [42] at the level of contactus adherents, a special type of plaque-bearing adhering

junction containing M-cadherin, in the granule cell layer of the cerebellar glomerulus.

N-cadherin immunoreactivity at the level of Lugaro cells is apparently related with the dendro-somatic and somato-somato contacts between Lugaro cells, and dendro-dendritic contacts between Lugaro and Golgi and basket cells, and their synaptic contacts with other cerebellar nerve cells [27, 43, 44].

Neurobiological Considerations on Lugaro Cells

Dieudonné [45] postulated that serotonin specifically modulates the activity of Lugaro cells, a class of inhibitory interneurons of the cerebellar cortex, offering new insights on the action of this neuromodulator. The peculiar axonal projection and specific interneuronal targets of the Lugaro cells suggest that the action of serotonin might occur up stream of Purkinje cells through a resetting of the computational properties of the cerebellar cortex. Dean et al. [46] recorded a novel fast GABAergic synaptic current from Lugaro cells to Purkinje cells in rat brain slices using patch-clamp techniques. The Authors suggested that the release of GABA onto Purkinje cells from Lugaro cells would primarily occur during motor activity under conditions in which the activity of basket and stellate cells might be inhibited. Melik-Musyan and Fanardzhyan [9,43,44] emphasized that the processes of Lugaro cells have very large spatial expansion and form numerous axosomatic and axodendritic connections with all neurons and fibers of the cerebellar cortex. Structural and topographic characteristics of Lugaro cells, as well as the peculiarities of their contacts with the other cells of cerebellar cortex, in combination with the data on their neurotransmitter content, indicate that these cells play the role of inhibitory interneurons. Ito [47] considers that cerebellar circuitry now includes Lugaro cells and unipolar brush cells as additional unique elements in the neuronal machine concept of the cerebellum. Ambrosi et al. [48]

support the concept that Lugaro cells act as an inhibitory interneuron.

ACKNOWLEDGEMENTS

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