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PROXIMITY OF EXCITATORY SYNAPSES AND ASTROGLIAL GAP JUNCTIONS IN LAYER IV OF THE MOUSE BARREL CORTEX

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Abstract—Neurons and astrocytes, the two major cell populations in the adult brain, are characterized by their own mode of intercellular communication – the synapses and the gap junctions (GJ), respectively. In addition, there is increasing evidence for dynamic and metabolic neuroglial interactions resulting in the modulation of synaptic transmission at the so-called ''tripartite synapse''. Based on this, we have investigated at the ultrastructural level how excitatory synapses (ES) and astroglial GJ are spatially distributed in layer IV of the barrel cortex of the adult mouse. We used specific antibodies for connexin (Cx) 30 and 43 to identify astroglial GJ, these two proteins are known to be present in the majority of astroglial GJ in the cerebral cortex. In electron-microscopic images, we measured the distance between two ES, between two GJ and between a GJ and its nearest ES. We found a ratio of two GJ per three ES in the hollow and septal areas. Taking into account the size of an astrocyte domain, the high density of GJ suggests the occurrence of reflexive type, i.e. GJ between processes of the same astrocyte. Interestingly, the distance between an ES and an astroglial GJ was found to be significantly lower than that between either two synapses or between two GJ. These observations indicate that the two modes of cell-to-cell communication are not randomly distributed in layer IV of the barrel cortex. Consequently, this feature may provide the morphological support for the recently reported functional interactions between neuronal circuits and astroglial networks.

Key words: glia, astrocyte, connexins, somatosensory cortex.

INTRODUCTION

The two main brain cell populations, neurons and astrocytes, establish tight and dynamic interactions (Haydon and Carmignoto, 2006). Although electrical synapses based on GJ communication exist between some adult neurons (Pereda et al., 2013) including in the somatosensory cortex (Connors and Long, 2004), each of these two brain cell types are characterized by their specific modality of intercellular communication. Indeed, information processing is mainly achieved by chemical transmission at synapses between neurons, while gap junctions (GJ) support direct exchanges of ions and small signaling molecules between astrocytes (Giaume et al., 2010). Recently, it has been shown that astrocyte gap junctional communication is controlled by neurons in an activity-dependent manner (Roux et al., 2011) and that reversibly lack of GJ proteins in astrocytes increases synaptic transmission in CA1 pyramidal neurons (Pannasch et al., 2011). Based on these findings, it remains to be determined how the interplay between the two modes of communication of each brain cell type is organized at the ultrastructural level.

In their ultrastructural exploration of the lateral hypothalamic area of the adult rat, Sipe and Moore (1976) noticed that most (about 90%) of astrocyte GJ, identified on the basis of morphological criteria, were positioned within a distance of 15–20 μm from a synaptic terminal (Sipe and Moore, 1976). This feature was also outlined by Yamamoto and collaborators who mentioned that in the cerebellum ''*The adjacent neuropil was sparsely peppered with junctions 0.6 to 1 μm in length between processes which were often closely associated with chemical synapses or dendritic profiles*.'' (Yamamoto et al., 1990). Moreover, a quantitative analysis, based on electron microscopic reconstructions, gave an estimated number of 30,000–60,000 GJ on average *per* astrocyte in the visual cortex of the rat (see Rohlmann and Wolf, 1996).

Given the growing interest in neuron-glial interactions (Halassa and Haydon, 2010), these early observations

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Abbreviations: Cx, connexin; DAB, 3,3′-diaminobenzidine; ES, excitatory synapses; GJ, gap junctions; TEM, transmission electron microscopy.

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warrant further detailed analysis, especially because tools to target the two main molecular constituents of astroglial GJ channels are now available. Thus, the present study was undertaken in order to quantitatively establish by transmission electron microscopy (TEM) the spatial distribution of excitatory synapses (ES) and astroglial GJ in the somatosensory cortex where the functional properties of astroglial GJ were previously determined (Houades et al., 2008).

In the cerebral cortex, astroglial GJ are composed by two molecular constituents, connexin 43 and 30 (Cx43 and Cx30), which are not expressed in adult neurons (see Giaume et al., 2010). Double-labeling studies at the ultrastructural level have confirmed the presence of these two Cx in astroglial GJ within the CNS (Nagy et al., 1999). However, so far no study has specifically addressed their co-localization in astroglial GJ in layer IV of the adult mouse barrel cortex. Here we used a pre-embedding immunolabeling protocol for Cx43 and Cx30 to visualize astroglial GJ in layer IV of the barrel cortex using TEM. This protocol allows identifying clearly the sites where two labeled astroglial membranes form a GJ. However, the 3,3′-diaminobenzidine (DAB)-labeling slightly disrupts the ultrastructure at the level of the labeled membranes and therefore the description of a typical GJ, as seen with high-resolution TEM, characterized by a 2–4-nm inter-membrane spacing cannot be applied to the immuno-labeled material used for the current study. The histological procedure chosen here should be considered as a compromise between immuno-labeling and ultrastructural preservation of the tissue. It renders the recognition of non-(DAB)-labeled GJ very difficult. The stereological analysis was performed on sets of three serial, ultra-thin sections in which labeled GJ and ES could easily be identified on the basis of morphological criteria. Using a large dataset, our analysis showed the preferential localization of astroglial GJ in the vicinity of ES within the cerebral somatosensory cortex of the adult mouse. We also conclude that based on the number of GJ and taking into account the size and the notion of astrocytic domain (Bushong et al., 2002; Ogata and Kosaka, 2002), number of observed GJ has to occur between processes of the same astrocyte.

EXPERIMENTAL PROCEDURES

All experiments were performed according to the European Community Council Directives of 1st January, 2013 (2010/63/EU) and all efforts have been made to minimize the number of animals used as well as their suffering. In addition, procedures were reviewed and approved by the Office Vétérinaire Cantonal (Lausanne), in accordance with Swiss Federal Laws.

Animals and histological preparations

Adult mice ($n = 5$) of the NOR-strain (Van der Loos et al., 1986) were deeply anaesthetized with a lethal dose of Nembutal (160-mg/kg body weight; i.p.) and were fixed by transcardial perfusion with a 400 ml solution of 2% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1 M; pH 7.4). After one hour, brains were removed and postfixed in the same fixative for another hour. Hemispheres were oriented for being cut (60 μm) in a plane tangential to the pial surface of the barrel cortex using a vibratome (Leica VT100; Wetzlar, Germany). Sections containing the barrels in layer IV were selected for further processing using a dissecting microscope. They were rinsed in 0.1 M PBS (pH 7.4) and cryoprotected in a solution of 2% glycerin, 20% DMSO in 0.1 M PBS (pH 7.4) for 15 min and subsequently freeze–thawed twice in liquid nitrogen to improve the immunolabeling. After rinsing three times in the PBS solution, sections were pretreated in 0.3% hydrogen peroxide in PBS for 10 min.

Prior to first antibody exposure, sections were blocked in PBS containing 5% NGS and 0.1% BSA-c (Aurion, The Netherlands), three times, 10 min for each rinse, and then incubated overnight in PBS (0.1 M, pH 7.4; 0.1% BSA-c) at 4 °C with the following primary antibodies: one third of the sections exposed to a monoclonal antibody for Cx43 (BD Bioscience, USA) at a dilution of 1:1000; one third, using a rabbit-raised polyclonal antibody for Cx30 (Chemicon, Germany) diluted at 1:500 and the final third of the sections was incubated without addition of an antibody. After washing, the sections were exposed to the biotinylated secondary antibody [1/400 goat-anti-rabbit or goat-anti-mouse Fab fragment, Jackson Laboratories, USA], in the PBS containing 5% NGS and 0.1% BSA-c and left for 2 h before being washed in TBS (0.1 M, pH 8.0), incubated in avidin–biotin peroxidase complex (ABC Elite, Vector Laboratories, USA) for 2 h, and finally incubated in 0.04% of 3,3′-diaminobenzidine tetrachloride (Fluka, Switzerland) and 0.015% H_2O_2 in the TBS solution for 25 min. Sections were then washed in TBS, three times in cacodylate buffer (0.1 M, pH 7.4) and postfixed in 1% osmium tetroxide in cacodylate buffer (0.1 M). Subsequently they were dehydrated in alcohol and flat-embedded between silicon-coated glass slides in Durcapan ACM resin (Fluka, Switzerland). Once the resin had cured 48 h at 60 °C, the cortical barrels could be identified on the 60-μm embedded sections and a piece of tissue was cut such that it included the septum between barrels B2 and C2 in its middle, flanked by the hollow of these barrels. Camera-lucida drawings were made of the block prior to cutting to allow the identification of the barrel regions in the thin sections using blood vessels as landmarks. Series of Silver/gray (60-nm thickness) thin sections were cut using an ultramicotome (Reichert, Ultracut E, Austria) and placed on formvar-coated, single-slot, gold grids. Images were taken using a Phillips CM12 electron microscope at 80 kV (magnification 7900–13,500×) and a digital camera Megaview III (Olympus, Japan).

Analysis

From each of the two series of immunolabeled sections (Cx30 and Cx43) per animal, 15 × 3 serial images were taken in the hollow and in the septum of the posterior part of the barrel cortex; i.e. the part with the large barrels. As a first step, a semithin section was cut from the surface of the block and stained with Nissl Blue. The observation of this section with a light microscope