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DEVELOPMENTAL CHANGES IN THE FLOTILLIN-1 EXPRESSION PATTERN OF THE RAT VISUAL CORTEX

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Abstract—Ocular dominance plasticity is activity dependent, changes in response to eye competition, and is transitory during developmental stages. Lipid rafts have modulatory functions in cellular, physiological, and behavioral processes. Although many of these modulatory roles are mediated by flotillin-1, a lipid raft-associated protein, the ontogenetic changes in the cellular and subcellular distribution patterns of flotillin-1 are unclear. I investigated the developmental pattern of the distribution of flotillin-1 in the rat visual cortex with immunohistochemistry at both light and electron microscopic levels. An affinity-purified anti-flotillin-1 antibody reacted with a single band of about 40–50 kDa in total proteins prepared from the rat visual cortex. Flotillin-1 levels transiently increased on postnatal days 21–35. Flotillin-1 immunoreactivity at 3 weeks of age was broadly distributed though all visual cortical layers, but it exhibited a relatively higher density in layers II/III and V/VI. Flotillin-1 immunoreactivity at 3 months of age was significantly decreased compared with that at 3 weeks of age. Strong flotillin-1 immunoreactivity was observed in both neuronal perikarya and processes at 3 weeks of age. Double-labeling experiments with anti-microtubule-associated protein 2, anti-neurofilament, anti-synaptophysin, anti-vesicular glutamate transporter 1, anti-vesicular glutamate transporter 2, anti-glial fibrillary acidic protein, and flotillin-1 mainly labeled the somata of excitatory neurons and corticocortical synapses. Some flotillin-1 was distributed in excitatory neuron axons, thalamocortical synapses, astrocytes, oligodendrocytes, and microglial cells. Immunoelectron microscopy revealed numerous regions of flotillin-1 immunoreactivity near the rough endoplasmic reticulum in neurons and presynaptic regions at 3 weeks of age. These findings illustrate early developmental changes in the cellular and subcellular localization of flotillin-1 protein in the rat visual cortex. Moreover, the ultrastructural distribution of flotillin-1 immunoreactivity suggested that flotillin-1 was transported mainly into presynaptic terminals where it exerts effects at the presynaptic sites of excitatory and inhibitory neurons.

Key words: flotillin-1, lipid raft, immunohistochemistry, immunoelectron microscopy, synaptic plasticity.

INTRODUCTION

Neuronal development is important for learning and memory. After neurite outgrowth, neurites begin to form synapses with other neurons to promote the formation of the neuronal circuitry. Synapses, which are necessary for interactions with environmental stimuli, are added and removed before they mature. Activity-dependent changes in synaptic plasticity play an important role in the central nervous system during the development (Kaminska et al., 1995; Hensch et al., 1998; Lamsa and Taira, 2003; Nudo, 2003; Weber et al., 2003; Thakur et al., 2004). Ocular dominance (OD) plasticity has been examined in developmental animal models following monocular deprivation in the visual cortex (Wiesel et al., 1974; Hubel et al., 1977; Issa et al., 1999; Levelt and Hubener, 2012). OD plasticity is activity dependent, changes in response to competition in both eyes, and is transitory during developmental stages. Monocular deprivation normally alters OD in the rat visual cortex during the postnatal critical period (postnatal days 17–45) (Maffei et al., 1992; Fagiolini et al., 1994). The noradrenergic (NA) system plays important roles in OD plasticity through beta-1 adrenoceptors (Kasamatsu and Pettigrew, 1976, 1979; Kasamatsu et al., 1979; Kasamatsu and Shirokawa, 1985; Shirokawa and Kasamatsu, 1986; Imamura and Kasamatsu, 1989, 1991; Shirokawa et al., 1989). Furthermore, in the developing visual cortex, the NA system, which acts through alpha-1 adrenoceptors, plays important roles in the synapse formation (Blue and Parnavelas, 1982), synaptic plasticity (Kirkwood et al., 1999; Inaba et al., 2009), and maintenance of excitatory synapses (Nakadate et al., 2006). The activation of alpha-1 adrenoceptors selectively suppresses the horizontal propagation of excitation in the supragranular layers of the rat visual cortex (Kobayashi et al., 2000). These receptors that are related to the modulation of synaptic plasticity have also been shown to be distributed on lipid rafts (Chini and Parenti, 2004).

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Abbreviations: CNPase, 2-3-cyclic nucleotide 3-phosphodiesterase; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; OD, ocular dominance; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.3% Triton X-100; RT, room temperature; SDS, sodium dodecyl sulfate; TPBS, PBS containing 0.1% Tween 20.

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In addition to the regulation of synaptic formation with receptors on lipid rafts, it is possible that lipid rafts themselves influence the synapse formation. Lipid rafts are microdomains of the plasma membrane that are rich in glycosphingolipids, cholesterol, and acylated proteins and act as signaling platforms (Pike, 2006). Many receptors that are expressed on lipid rafts (Chini and Parenti, 2004) are involved in various cellular functions, such as signal transduction, molecular sorting, and membrane trafficking, through each of these expressed receptors (Simons and Ikonen, 1997; Lafont et al., 1999). The cholesterol that is derived from glial cells induces the formation of synapses (Mauch et al., 2001). By increasing the number of lipid rafts, the cholesterol synthesis within neurons can enhance synaptogenesis (Suzuki et al., 2007). In addition, intact lipid rafts (Hering et al., 2003) and cholesterol homeostasis (Goritz et al., 2005) are necessary for synapse stability.

Flotillin-1, which is a lipid raft-associated protein, has been shown to be important in the early stages of neuronal development (Carcea et al., 2010; Swanwick et al., 2010a,b). Flotillin-1 was identified as a caveolae-associated integral membrane protein in the brain and the lung (Bickel et al., 1997). Although neurons do not express caveolin or possess caveolae, flotillin-1 is abundant in the brain. It is expressed in pyramidal neurons and astrocytes in the human brain tissue (Kokubo et al., 2000). Flotillin-1 is a 428-amino-acid protein that is associated with the cytoplasmic side of lipid raft membranes (Solis et al., 2007). It is ubiquitously expressed in all cell types in adulthood (Morrow and Parton, 2005), and the flotillin-1 expression increases during developmental stages (Volonte et al., 1999). Flotillin-1 induces filopodia formation in neurons (Hazarika et al., 1999; Neumann-Giesen et al., 2004) and neuritic outgrowth (Swanwick et al., 2010a) as well as promotes hippocampal neuronal differentiation (Munderloh et al., 2009; Swanwick et al., 2010b).

Previous studies of the distribution of flotillin-1 have mainly been conducted with biochemical or immunofluorescence microscopy methods in cultured cells. Moreover, the subcellular localization of flotillin-1 in tissues has not been demonstrated. Therefore, I investigated the localization of flotillin-1, the lipid raft-associated protein, to clarify developmental changes. In the present study, I performed an immunoelectron microscopic analysis of flotillin-1 and double immunostaining of flotillin-1 and various markers to investigate developmental changes in the subcellular localization of flotillin-1 in the rat visual cortex around the time of the critical period of OD plasticity.

EXPERIMENTAL PROCEDURES

Animals

In this study, 30 Sprague–Dawley male rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used. The animals were housed under temperature- and humidity-controlled conditions with a 12-h light/dark cycle and *ad libitum* access to food and water. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the animal research committee of the Meiji Pharmaceutical University. In the present study, all efforts were made to minimize the suffering of animals and to reduce the number of animals used.

Antibody for flotillin-1

A polyclonal antibody to flotillin-1 was purchased from Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan). It had been raised against the C-terminal part of the common site of the rat and mouse flotillin-1 sequence (SQVNHNKPLRTA). This sequence is 100% conserved in the rat and mouse flotillin-1. To examine the specificity, antibodies were preadsorbed with synthetic peptides that were based on the findings of the epitope mapping. In brief, 100 μg of the synthetic peptide (SQVNHNKPLRTA) was incubated with 10 μg of the anti-flotillin-1 polyclonal antibody at 4 °C for 24 h on a rotation vortex. The supernatant solution containing the blocked antibody was then tested with immunoblotting and immunohistochemical analyses, as described below.

Protein preparation for western blotting

Proteins were prepared for western blotting in accordance with methods described previously (Nakadate et al., 2006; Amaddii et al., 2012). Rats of each age were perfused through the left ventricle with ice-cold saline. The brains were rapidly removed and homogenized in 10 volumes of ice-cold homogenate buffer (20 mM Tris–HCl at pH 7.5, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 150 mM NaCl) containing protease inhibitors (one tablet/10 mL homogenate buffer, Complete™ Mini, Roche Diagnostics, Basel, Switzerland). Homogenates were then centrifuged at 500*g* for 5 min at 4 °C, and supernatants were dissolved in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 3% SDS, 5% glycerol, and 2% 2-mercaptoethanol) and then boiled for 5 min. According to the Bradford method, protein concentrations were measured with a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and determined with bovine serum albumin, as previously described (Nakadate et al., 2006).

Immunoblotting

SDS–polyacrylamide gel electrophoresis and western blotting were performed with the ECL-Plus immunoblotting detection system (GE Healthcare Life Sciences, Buckinghamshire, UK), as previously described (Nakadate et al., 2006). The proteins were separated by SDS–polyacrylamide gel electrophoresis (12% gels) and electrophoretically transferred at 50 V for 60 min onto a polyvinylidene difluoride membrane (Immobilon™-P, EMD Millipore, Billerica, MA, USA). After blocking with 5% (w/v) skim milk (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and Block-Ace