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POSTSYNAPTIC ENRICHMENT OF Eps8 AT DENDRITIC SHAFT SYNAPSES OF UNIPOLAR BRUSH CELLS IN RAT CEREBELLUM

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Abstract—Epidermal growth factor receptor pathway substrate 8 (Eps8) is a widely expressed multidomain signaling protein that coordinates two disparate GTPase-dependent mechanisms: actin reorganization via Ras/Rac pathways and receptor trafficking via Rab5. Expression of *Eps8*, the gene encoding the founding member of the Eps8 family of proteins, was found in cerebellum by virtual Northern analysis and *in situ* hybridization. Because the cerebellum has a well-known cellular architecture and is a favored model to study synaptic plasticity and actin dynamics, we sought to analyze Eps8 localization in rat cerebellar neurons and synapses by light and electron microscopy.

Specificity of Eps8-antibody was demonstrated by immunoblots and in brain sections. In cerebellum, unipolar brush cells (UBCs) were densely Eps8 immunopositive and granule cells were moderately immunostained. In both types of neuron immunoreaction product was localized to the somatodendritic and axonal compartments. Postsynaptic immunostained foci were demonstrated in the glomeruli in correspondence of the synapses formed by mossy fiber terminals with granule cell and UBC dendrites. These foci appeared especially evident in the UBC brush, which contains an extraordinary postsynaptic apparatus of actin microfilaments facing synaptic junctions of the long and segmented varieties. Eps8 immunoreactivity was conspicuously absent in Purkinje cells and their actin-rich dendritic spines, in all types of inhibitory interneurons of the cerebellum, cerebellar nuclei neurons, and astrocytes. In conclusion, Eps8 protein in cerebellum is expressed exclusively by excitatory cortical interneurons and is intracellularly compartmentalized in a cell-class specific manner. This is the first demonstration of the presence of a member of the Eps8 protein family in UBCs and its enrichment at postsynaptic sites. **© 2006 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: actin, granule cells, cerebellum, synaptic junction, Purkinje cell.

Epidermal growth factor receptor substrate 8 (Eps8), the founding member of the Eps8 family of proteins, links two important GTPase-mediated functions, i.e. actin reorganization via the Ras/Rac pathway and epidermal growth factor receptor (EGFR) internalization via Rab5, by forming multiprotein complexes (reviewed in Di Fiore and Scita, 2002). Eps8 is able to form these macromolecular complexes because like other signaling molecules, it has multiple motifs that include: 1) a proline-rich domain; 2) a phosphotyrosine binding (PTB) domain; 3) an SH3-binding domain which mediates binding with Abi-1 (Interacting Protein-1) (Biesova et al., 1997; Agrawal et al., 2002) and RN-tre (Lanzetti et al., 2000); 4) a binding region for EGFR; and 5) a C-terminal effector region. Apart from its ability to bind actin and regulate actin polymerization by itself (Disanza et al., 2004, 2005), the C-terminal effector region also mediates a weak but activating interaction with Sos-1 (Son of Sevenless-1) (Scita et al., 2001; Innocenti et al., 2002) and has a barbed-end capping activity independent of Rac (Disanza et al., 2004, 2005).

By virtual Northern analysis, human *EPS8* and the related gene *EPS8R2* were found to be expressed in cerebellum (Tocchetti et al., 2003). In the Gensat (http://www.ncbi.nlm.nih.gov) and Allen brain atlas databases (http://www.brain-map.org), Eps8 expression is present in the developing and mature rodent cerebellum. Because the cerebellum has a well-known cellular architecture and is a favored model to study synaptic plasticity (Ito, 1984) and actin dynamics (Dunaevsky and Mason, 2003; Matus, 2005), we sought to analyze Eps8 localization in cerebellar neurons and synapses.

The three-layered cerebellar cortex contains seven neuron classes, the Purkinje, stellate, basket, granule, unipolar brush (UBC), Golgi and Lugaro cells, of which all but the Purkinje cell are local circuit neurons. The granule cells (Eccles et al., 1967) and the UBCs (Nunzi et al., 2001) are excitatory neurons, while all others classes, including the Purkinje cell, are inhibitory (Eccles et al., 1967).

In the cerebellum, as everywhere else in the brain, neurons communicate primarily through chemical synapses. The structural and functional properties of synaptic junctions are generally cell class-specific and are often conserved across species. Synaptic junctions are in a dynamic state, as the activity of the synapse changes ionic fluxes and elicits complex interactions between receptors, scaffolding and regulatory proteins (reviewed in Dunaevsky and Mason, 2003; Carlisle and Kennedy, 2005; Nicholson-Dykstra et al., 2005; Oertner and Matus, 2005; Segal, 2005; Tada and Sheng, 2006).

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Abbreviations: BSA, bovine serum albumin; DAB, diaminobenzidine; EGFR, epidermal growth factor receptor; Eps8, epidermal growth factor receptor substrate 8; GFAP, glial fibrillary acid protein; IRSp53, insulin receptor tyrosine kinase substrate p53; mGluR1α, metabotropic glutamate receptor 1α; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline with 0.2% Triton X-100; UBC, unipolar brush cell.

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Actin is a major component of excitatory synapses (reviewed in Dillon and Goda, 2005); presynaptically, it is involved with the synapsins in regulating transport and deployment of synaptic vesicles (Halpain, 2003; Hilfiker et al., 2005) and postsynaptically it participates in short-term and long-term, activity-dependent synaptic plasticity that can change the shape and size of synaptic junctions and synaptic profiles (Star et al., 2002; Fukazawa et al., 2003). Actin is particularly enriched in neuronal spines, where it anchors postsynaptic glutamate receptors and regulates the formation, maintenance, shape and size of these dendritic protrusions through its interactions with a variety of actin-binding proteins (Ackermann and Matus, 2003; Okamoto et al., 2004; Ethell and Pasquale, 2005; Nicholson-Dykstra et al., 2005). Little is known about the actin cytoskeleton of the dendritic shafts, even though they bear excitatory synapses in neuron types provided with few spines or lacking spines altogether.

In the cerebellar cortex, there are four main classes of excitatory synapses, two of which are situated in the molecular layer and two in the granular layer: 1) the synapses of climbing fibers on dendritic spines of the proximal Purkinje cell arbor; 2) the synapses of parallel fibers on dendritic spines of the distal Purkinje cell arbor; 3) the synapses of mossy fibers on the finger-like tips of granule cell dendrites in the granular layer glomeruli; and 4) the synapses of mossy fibers on the dendrioles of UBC brushes in a subset of granular layer glomeruli (Mugnaini, 1972; Palay and Chan-Palay, 1974; Rossi et al., 1995; Mugnaini et al., 1997). While Purkinje spine synapses and granule cell synapses are homogeneously distributed throughout the cortex, UBC synapses occur at high density in the vestibulo-cerebellar lobe and at lower densities in the vermis, but are rare or absent in the lateral portions of the cerebellar hemispheres. The four classes of excitatory contacts differ in size of the synaptic junctions and in the amount of postsynaptic actin they contain. The synapses between climbing fibers and parallel fibers and Purkinje cell spines are small patches, about 0.2 μm in diameter (Mugnaini, 1972), and the spines are endowed with a diffuse complement of actin microfilaments (Landis et al., 1987; Dunaevsky and Mason, 2003; Matus, 2005). The synapses between mossy fibers and granule cell dendritic tips are even smaller spots, measuring 0.1 μm in diameter (Morin et al., 2001); the dendritic tips resemble spines in so far as they are phalloidin-positive and have a diffuse actin cytoskeleton (Diño and Mugnaini, 2000; Capani et al., 2001a,b). In contrast, mossy fiber synapses on UBC brushes range from 0.2 μm to over 1 μm in diameter, and display an array of bundled postsynaptic actin filaments linking the postsynaptic density with the core of the brush dendriole (Diño and Mugnaini, 2000).

In this study we analyze the presence of Eps8 protein in neurons and synapses of the rat cerebellum utilizing immunocytochemical, light and electron microscopic methods.

EXPERIMENTAL PROCEDURES

Animals and perfusion

Young (26 day-old) Wistar albino males and adult (200–250 g of body weight) Sprague–Dawley rats of both sex were purchased from a commercial breeder (Harlan, Indianapolis, IN, USA) and processed according to approved guidelines. For immunocytochemistry, adult rats were deeply anesthetized with i.p. sodium pentobarbital (60 mg/kg body weight) and perfused through the ascending aorta with appropriate fixatives. For light microscopic immunocytochemistry, adult rats (N=5) were perfused with saline followed by 200 ml or 500 ml of 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were cryoprotected in 30% sucrose dissolved in phosphate-buffered saline (PBS). Frozen sections, 30 μm thick, were cut in the sagittal and coronal planes on a freezing stage microtome. For immunoelectron microscopy, adult rats (N=2) were perfused with saline followed by a fixative containing 4% freshly depolymerized paraformaldehyde in 0.1 M PB. After postfixation for 1 h at 4 °C, cerebella were removed and sliced on a Vibratome at 50 μm in the coronal or sagittal plane. For intracellular injection, young Wistar albino rats were placed under deep anesthesia with isoflurane (0.4 ml in 1 l, administered for ~2 min) and decapitated. The cerebella were quickly removed and placed into a "cutting" solution (125 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM Na_2HPO_4, 25 mM glucose, 0.2 mM $CaCl_2$, 7 mM $MgCl_2$, 1 mM kynurenic acid, and bubbled with 95% O_2/5% CO_2), and parasagittal slices (300 μm-thick) of the cerebellar vermis were cut using a vibrating blade microtome (Ted Pella Inc, Redding, CA, USA). After equilibration in an oxygenated solution, slices were mounted in a patch-clamp recording rig.

All experiments conformed to protocols approved by the Northwestern University Animal Care and Use Committee (ACUC). We followed guidelines issued by the National Institutes of Health and the Society for Neuroscience to minimize the number of animals used and their suffering.

Immunocytochemistry

For bright field microscopy sections were processed for immunocytochemistry according to an avidin/biotin amplification protocol. Briefly, the endogenous peroxidase activity was blocked in 0.3% H_2O_2 and 10% methanol in Tris-buffered saline (TBS; 100 mM Tris, 150 mM NaCl; pH 7.4). Unspecific binding was suppressed in a blocking solution containing 3% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in Tris-buffered saline with 0.2% Triton X-100 (TBS-T). Sections were then incubated with mouse anti-Eps8 monoclonal antibody (1:1000; BD Biosciences, San Jose, CA, USA), followed by incubation with biotinylated anti-mouse IgG (1:500; Amersham, Piscataway, NJ, USA) in 1% NGS/1% BSA/TBS-T. After rinsing in TBS, sections were incubated with avidin/biotin using ABC Elite kit (Vector, Burlingame, CA, USA) and visualized by diaminobenzidine (DAB) reaction.

For immunofluorescence sections were blocked with 3% NGS/1% BSA/TBS-T followed by incubation with the primary antibodies: mouse anti-Eps8 (1:2000); rabbit anti-GABA (1:2000; Sigma, St. Louis, MO, USA); rabbit anti-glial fibrillary acidic protein (GFAP; 1:2000; Dako, Carpinteria, CA, USA); rabbit anti-calretinin (1:1000; Chemicon, Temecula, CA, USA); rabbit anti-metabotropic glutamate receptor 1α (mGluR1α; 1:400; from Dr. R. Shigemoto, SORST-JST, Myodaiji, Okazaki, Japan); rabbit anti-synaptophysin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-espin (1 μg/ml; Sekerková et al., 2003). Bound primary antibodies were visualized by secondary antibodies coupled to Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR, USA). F-actin was visualized using Alexa 488-phalloidin (Molecular Probes). Sections were mounted with Vectashield (Vector).

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