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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 a family 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 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 somato-dendritic 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.

Keywords

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-

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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 in situ hybridization and virtual Northern analysis, *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).

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;Fuzakawa 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 (200–250g of body weight) Sprague-Dawley rats of both sex were purchased from a commercial breeder (Harlan, Indianapolis, IN) and processed according to approved guidelines. For immunocytochemistry, adult rats were deeply anesthetized with intraperitoneal sodium pentobarbital (60mg/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 or 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, 30μm thick, were cut in the sagittal and coronal planes on a freezing stage microtome. For immuno-electron microscopy, adult rats (N=2) were perfused with saline followed by a fixative containing 4% freshly depolymerized paraformaldehyde in 0.1M PB. After postfixation for 1 h at 4°C, cerebella were removed and sliced on 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 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 TBS with 0.2% Triton X-100 (TBS-T). Sections were then incubated with mouse anti-Eps8 monoclonal antibody (1:1000; BD Biosciences, San José, CA), followed by incubation with biotinylated anti-mouse IgG (1:500; Amersham, Piscataway, NJ) in 1% NGS/1% BSA/TBS-T. After rinsing in TBS, sections were

incubated with avidin/biotin using ABC Elite kit (Vector, Burlingame, CA) 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-gamma-amminobutyric acid (GABA; 1:2000; Sigma, Saint Louis, MO); rabbit anti-glial acidic protein (GFAP; 1:2000; DAKO, Carpinteria, CA); rabbit anti-calretinin (1:1000; Chemicon, Temecula, CA); rabbit anti-metabotropic 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); rabbit anti-espin (1μg/ml; Sekerková et al., 2003). Bound primary antibodies were visualized by secondary antibodies coupled to Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR). F-actin was visualized using Alexa 488-phalloidin (Molecular Probes). Sections were mounted with Vectashield (Vector).

For immuno-electron microscopy, free-floating Vibratome slices were processed with mouse anti-Eps8 antibody (1:1000) using avidin/biotin amplification protocol and DAB as the chromogen. Immunoreacted sections were postfixed in buffered 2% OsO_4, rinsed and stained in 1% uranyl acetate, and then dehydrated and embedded in Epon as described (Floris et al., 1994). Ultrathin sections were contrasted with lead citrate and analyzed under a Zeiss EM-10 electron microscope operated at 60–80 kV.

For all experiments, control sections incubated without the appropriate primary antibody lacked immunoreaction signal.

Post-hoc visualization of individual UBCs

Visualization of individual UBCs was achieved by filling the cells with biocytin (Sigma) during a typical patch clamp recording (Martina et al., 2000). UBCs were identified by their unique electrical properties (Russo et al., 2006). Cells were filled through the recording pipette, with 5% biocytin dissolved in the internal recording solution (140 mM K-gluconate, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 2 mM Na_2ATP, 0.1 mM NaGTP, pH 7.3 was adjusted with KOH). After allowing sufficient time for the internal solution to diffuse into all processes of the patched UBC (15–20 minutes), the pipette was removed, and slices were fixed in 4% paraformaldehyde in 0.1 M PB for 30 min at 20–22ºC. After fixation, slices were rinsed several times in PB, blocked in 1% BSA/0.5 % Triton X/PBS and incubated in mouse anti-Eps8 (1:1000) in 1% BSA/0.5 % Triton X/PBS for 3 days at 4ºC. After several PBS rinses the bound primary antibody and the biocytin were visualized by Alexa 568-coupled goat anti mouse IgG (1:500; Molecular Probes) and either FITC-labeled streptavidin (1:200; Jackson ImmunoResearchs Laboratories, West Grove, PA) or Oregon green 488-coupled neutravidin (1:500, Molecular Probes).

Photography and image processing

Bright field and immunofluorescence images were acquired with a Spot RT CCD video camera (Diagnostics Instruments, Sterling Height, MI) mounted on a Nikon Eclipse E800 microscope. Laser scanning confocal analysis was performed with a Nikon PCM 2000 Confocal Microscope System to analyze immunofluorescence images. For colocalization experiments type DF immersion oil (Fryer, Huntley, Il) was used with either a 40x plan-flour lens (numerical aperture 1.3) or a 60x plan-apochromatic lens (numerical aperture 1.4). To minimize spillover between the channels the images were sequentially acquired and saved as tiff files with 150 pixels/inch resolution. Electron micrographic negatives were scanned and imported into Adobe Photoshop CS (Adobe Systems Inc.). All images were processed with Adobe Photoshop. RGB images were converted to CMYK color format with small adjustment of brightness and contrast. The

inset in Fig. 2L was further processed to filter out the background staining by adjusting the tonal range of the image.

RESULTS

Distinct Eps8 antibody staining was present in many regions of the gray matter, including olfactory bulb, anterior olfactory nuclei, basal forebrain, cerebral cortex, hippocampus, septal nuclei, amygdala, thalamus, hypothalamus, colliculi, pontine nuclei, cerebellum, cochlear nuclear complex and inferior olive, while the white matter was generally unstained (Fig. 1a, A, F). In most regions, Eps8 staining appeared diffuse, suggesting a predominant localization to cell processes in the neuropil (Fig 1). The cell bodies of either projection neurons or local circuit neurons, however, were immunostained to various degrees of intensity in several centers (not shown), including the posterior septal nuclei, globus pallidus, thalamus, hypothalamus, hippocampus, cerebral cortex, and cerebellar cortex. Here we focused on the cerebellum, and the full description of Eps8 labeling in brain will be detailed in a separate study.

Eps8 immunostaining in cerebellum

In the cerebellum, the molecular layer was moderately Eps8-immunostained throughout the cortex. The granular layer was faintly immunostained in most of the hemispheral and vermal cortex, while it was intensely stained in the caudal cerebellum. The intense immunostaining, which occurred in lobules IX-v (ventral uvula) and X (nodulus) (Fig. 1a, B), in the flocculus, and in the part of the ventral paraflocculus continuous with the flocculus (Fig. 1E), was localized to UBCs. The UBC somata (8–10 μm in diameter; arrowheads in Fig. 1C, D) were moderately stained and emitted a single dendrite terminating with an intensely stained brush (arrow in Fig. 1C, D). Immunostained UBCs were also present in the cochlear nuclear complex (Fig. 1F), in accordance with the presence of a cerebellum-like microcircuit in this region (Berrebi and Mugnaini, 1991;Mugnaini et al., 1997;Oertel and Young, 2004). In specimens fixed with only 200 ml cold formaldehyde immunostaining was apparent in the clustered small granule cell bodies and the glomeruli (discernible in the granule cell layer of Fig. 2D) throughout the cerebellum. These patterns suggested that Eps8 was present at high abundance in the somatodendritic compartment of the UBCs and at relatively lower abundance in both the somato-dendritic (granular layer) and axonal (molecular layer parallel fibers) compartments of the granule cells.

To ascertain whether Eps8 immunoreactivity in the molecular and granular layer was localized, at least in part, to the astroglia, we used two-color immunofluorescence with mouse anti-Eps8 and rabbit anti-GFAP antibodies. All cells of the astroglial family, i.e. Golgi-Bergmann glia, velate astrocytes of the granular layer and fibrous astrocytes of the white matter were Eps8-negative (Fig. 2A–C). We also used double-immunofluorescence staining with mouse anti-Eps8 and rabbit anti-GABA or rabbit anti-calretinin to ascertain whether Eps8 was localized to any of the inhibitory neurons, i.e., the Purkinje cells, stellate and basket cells, Golgi cells and Lugaro cells –all of which are known to use GABA neurotransmission (Mugnaini, 2000); rat Lugaro cells, in addition are calretinin-positive (Diño et al., 1999). In the cerebellar cortex, Eps8 immunostaining was absent from cerebellar inhibitory neurons of all types (Fig. 2D–I). Notably, the branches of basket cell axons surrounding the Purkinje cell axons (pinceaux) were also Eps8-negative.

Because the molecular layer staining was localized to densely packed, small, evenly distributed puncta that could presumably correspond not only to the varicose parallel fibers, which are the axons of the granule cells, but also to the spines of the Purkinje cell's distal dendrites, we used double immunostaining with mouse anti-Eps8 and an affinity purified rabbit antiserum to espin, an actin-bundling protein present in Purkinje cell spines (Sekerková et al., 2003). Binding sites of the two antibodies appeared distinct in individual, 0.5 μm thick, optical sections obtained

by confocal microscopy (Fig. 2J–L), thus excluding any immunopositivity of the Purkinje spines. The Eps8 antiserum did not reveal the terminal arborizations of the climbing fibers, which form powerful excitatory synapses on the proximal dendrites of the Purkinje cells, although the entire territory of the inferior olivary complex, from which these fibers originate, displayed a neuropil staining pattern. In the cerebellar nuclei Eps8 immunostaining seemed limited to the neuropil in both the large-celled (medial nucleus) and small-celled (ventral part of lateral nucleus) regions (panels A and E of Fig 1). However, there were distinctly Eps8-positive small cells (not shown) scattered along the ventricular surface that we identified as ectopic UBCs. The nature of the immunostaining in the inferior olive and the cerebellar nuclei was not analyzed further.

Specificity of the Eps8 antibody was assessed in immunoblots and brain sections. In Western blots, lanes for cerebellum, hippocampus, cortex, and brainstem showed a single Eps8-immunostained band, which was absent when the primary antibody was omitted (Fig. 1G). The Eps8 antiserum detected an ~97kDa protein, that matched the predicted molecular weight of Eps8 protein (Fazioli et al., 1993). Notably, we did not detect the minor 68 kDa protein that was reported on blots from NIH-EGFR cells (Fazioli et al., 1993). Brain sections processed without the primary antibody according to avidin/biotin amplification protocol were free of immunoreaction product (not shown).

Immunolocalization of Eps8 in granule neurons and mossy fibers

To verify the immunolocalization of Eps8 in the somato-dendritic and axonal compartments of granule cells, which are distributed in two different cortical layers, the granular and molecular layers, we then analyzed Eps8 immunoreactivity of these microneurons by pre-embedding immuno-electron microscopy (Fig. 3). The thin rim of perinuclear cytoplasm in granule cell bodies was lightly immunoreactive (data not shown). Immunolocalization of Eps8 was confirmed in granule cells axons forming the parallel fibers of the molecular layer (Fig. 3A) and their vesicle-filled varicosities (Fig. 3B). In the cerebellar glomeruli situated near the surface of the tissue slice, were processes are adequately accessed by immunoreagents, virtually all the digitiform tips of granule cell dendrites surrounding mossy fiber terminals were immunopositive (Fig. 3C), matching the pattern of fluorescence staining with phalloidin (Diño and Mugnaini, 2000;Capani et al., 2001a,b). The short postsynaptic densities of the mossy fiber-granule cell synapses were marked by DAB reaction product (but see Discussion).

In the caudal cerebellar folia we encountered immunoreactive mossy terminals (Fig. 3D), while in other cerebellar regions the mossy fiber terminals were immunonegative (mf in Fig. 3C). However, we could not establish whether the Eps8-positive mossy terminals represented intrinsic mossy fibers formed by UBC axons or belonged to a subclass of extrinsic mossy fiber afferents due to the absence of distinguishing ultrastructural criteria.

Immunolocalization of Eps8 in UBCs

Contrary to granule neurons, which constitute a homogeneous cell population and have somatodendritic and axonal compartments that are distributed in separate cortical layers, UBCs are heterogeneous and both their compartments are situated in the granular layer. Localization of Eps8 immunoreactivity to UBCs, therefore, required more elaborate light and electron microscopic approaches.

UBCs have been shown to fall in two categories, based on their chemical properties (Nunzi et al., 2002,2003) and time of origin (Sekerková et al., 2004). These two groups can readily be distinguished using calretinin and mGluR1α as cellular markers (Nunzi et al., 2002). To analyze the subcellular distribution of Eps8 in UBCs and determine whether the protein is differentially expressed in the two UBC subsets or represents a shared chemical phenotype common to all

UBCs, we employed confocal immunofluorescence microscopy using mouse monoclonal Eps8 antiserum in combination with rabbit antisera raised either against calretinin or mGluR1α. Eps8 immunofluorescence appeared moderate in the cell bodies and dendritic stems, and occurred mostly in the form of intensely positive foci (hereafter termed “hotspots” for simplicity) in the dendritic brushes (Fig. 4A, D). The Eps8 hotspots varied considerably in shape and size, frequently appearing as multiple patches, less than 1 μm in length, and as sausage-shaped ellipsoids whose long axis ranged from 1 μm to over 4 μm. Both calretinin-positive (Fig. 4A–C) and mGluR1α-positive (Fig. 4D–F) UBCs were Eps8 immunoreactive. In the merged images, the Eps8 immunoreactive hotspots were obfuscated by their colocalization with calretinin (Fig. 4C), consistent with previous findings that calretinin is distributed diffusely in the UBC cytoplasm (Floris et al., 1994); on the contrary, the Eps8 immunoreactive hotspots were usually distinct from mGluR1α-immunoreactive areas (Figs. 4F–I). This observation is in keeping with the previous electron microscopic demonstration that mGluR1α is enriched in non-synaptic appendages emanating from the UBC dendrioles lateral to the mossy fiber synapse, but is absent from the postsynaptic densities of UBCs (Jaarsma et al., 1998).

The UBC axon meanders in the granular layer, where it branches in the vicinity of the parent cell body and forms axonal terminals that strongly resemble the terminals of cerebellar mossy fiber afferents. UBC axons, therefore, have previously been classified as “intrinsic” mossy fibers (Diño et al., 2000b;Nunzi et al., 2002). To ascertain the presence of Eps8 in the UBC axonal compartment we therefore turned to cell filling. We first unequivocally identified individual UBCs by patch-clamp recording of their characteristic electrophysiological properties as detailed elsewhere (Russo et al., 2006), and then filled them with biocytin (Fig. 5A). After fixation and double labeling by Eps8 antibody the UBCs were imaged by confocal laser scanning microscopy. Out of twenty filled cells, four had axon terminals situated near the surface of the slice that where sufficiently accessed by antibodies. The axons of the filled UBCs were faintly immunostained in the preterminal portions, while they showed distinct Eps8 labeling in the body of the axon terminals (Fig. 5B–D). This finding indicates that the category of Eps8-positive mossy terminals observed by immuno-electron microscopy belong, at least in part, to UBC axons (Fig. 3D).

Eps8 hotspots in UBC brushes co-localized with postsynaptic F-actin, as shown by phalloidin labeling (Fig. 5E–J). Besides marking endothelial cells in the wall of blood vessels, intense phalloidin staining consistently coincided with Eps8 hotspots in UBC brushes. Granule cell dendritic tips were also phalloidin labeled, although to a much lesser extent. The Eps8 hotspots in UBCs were often found apposing unstained regions of the brush area (arrows in Fig. 5G, J), which were most likely occupied by the presynaptic mossy fiber. To verify whether Eps8 hotspots are exclusively postsynaptic, we used double-label immunofluorescence microscopy combining mouse monoclonal antibody to Eps8 and rabbit antiserum to synaptophysin, a synaptic vesicle protein (De Camilli et al., 2000). Confocal images of numerous UBC brushes showed that Eps8 hotspots are closely apposed to binding sites of antibodies against the synaptic vesicle marker, with only minimal overlap in 0.5 μm thick optical sections (Fig. 5K–N). Next, we sought to determine the subcellular distribution of Eps8 in UBCs by pre-embedding immuno-electron microscopy. Electron dense Eps8 immunoreaction product was present in the postsynaptic region of UBC dendrioles apposed to mossy fiber endings, which were recognized by their numerous synaptic vesicles and mitochondria (Fig. 6). The postsynaptic densities of the synapses formed by mossy fiber terminals with the UBC dendrioles were marked by DAB immunoreaction product (but see Discussion). The region of cytoplasm extending for approximately 0.5 μm from the postsynaptic plasma membrane to the cytoskeletal core of the dendriole was also distinctly immnunostained. Both mossy fiber-UBC synapses of the individual, long, uninterrupted variety (Fig. 6B) and the multiple, shorter variety (Fig. 6C) were distinctly Eps8-positive. The shapes and sizes of the Eps8-positive hotspots in electron microscopic sections and in confocal immunofluorescence images were

closely matched (compare Fig. 4H with 6A, and Fig. 4I with 6C). Synapses of the Golgi axons with UBC dendrioles (Go in Fig. 6A) were unstained.

DISCUSSION

In this study we demonstrate that in the rat cerebellum Eps8 protein was restricted to the two populations of excitatory interneurons innervated by mossy fibers: the granule cells and the UBCs. We provide evidence by light and electron microscopic immunocytochemistry that Eps8 is present in both the somatodendritic and axonal compartments of granule cells and UBCs and is differentially distributed within these two cell types, as shown by its particular abundance in the actin-rich, glomerular postsynaptic sites belonging to the digitiform tips of granule cells and the dendrioles of the UBC brushes.

Selective expression of Eps8 in granule cells and UBCs

Our immunocytochemical data provide evidence for the expression of Eps8 protein in the rat brain previously suggested in the mouse brain by virtual Northern analysis (Toccheti et al., 2003) and in situ hybridization (Allen Brain Atlas and Gensat databases). Although hybridization signal is distinct throughout the cerebellar granular layer in both databases, enrichment of the signal in the caudal cerebellum is evident only in the Allen Brain Atlas. Our data further highlight the localization of the antigen to specific classes of cerebellar neurons.

By double labeling with Eps8 and cell class markers we showed that all other cerebellar cells, including the Purkinje cells, the inhibitory neurons of the cerebellar cortex (the stellate, basket, Golgi and Lugaro cells), the excitatory large neurons and the inhibitory small neurons of the cerebellar nuclei, and the cerebellar glia were consistently Eps8-immunonegative. The absence of Eps8 immunostaining in climbing fiber arborizations in all lobules of the cerebellar cortex and in mossy fiber terminals in the lobules that are devoid of UBCs pointed to a selective expression of Eps8 in the two classes of cerebellar excitatory interneurons. The cerebellar localization of Eps8, however, should not be taken to indicate that the protein is a universal marker of CNS excitatory interneurons, because certain subpopulations of GABAergic interneurons - still to be fully characterized in the anterior olfactory nuclei, hippocampus and cerebral cortex - were strongly Eps8-immunoreactive (our unpublished observations).

Our data indicating that immunostaining of granule cells is homogeneous throughout the cerebellum invites the conclusion that Eps8 expression is a general granule cell phenotype. Compared to granule cells, Eps8 immunostaining of UBCs is patently more intense. We have previously shown two distinct subpopulations of UBCs in the rodent and primate cerebellum, one that is labeled by the calcium binding protein, calretinin, and the other by the mGluR1α glutamate receptor isoform (Nunzi et al., 2002,2006). Because Eps8 immunoreaction is localized to both calretinin-positive UBCs and mGluR1α-positive UBCs, we propose that Eps8 expression is also a likely marker for the entire UBC population. The higher density of UBCs in vestibulocerebellar folia (lobules IX and X) compared to other cerebellar lobules (Diño et al., 1999;Nunzi et al., 2006) may indeed account for the relatively higher Eps8 mRNA signal apparent in the mouse brain sections of the Allen brain atlas within these folia, compared to the rest of the cerebellum. Thus, we would propose that specific tests of vestibulo-ocular function might reveal previously unexplored phenotypes of Eps8-knockout mice (Offenhauser et al., 2004,2006).

Compartmentalization of Eps8 protein

The differential distribution of pre- and postsynaptic Eps8 immunoreaction product in the somatodendritic, axonal, and postsynaptic compartments of granule cells and UBCs requires special consideration, as it is indicative of a probable multiplicity of Eps8 functions.

In analyzing the distribution of Eps8 in the axons of granule cells and UBCs, it should be noted that the axonal endings are spaced only 4–5 μm apart in the parallel fibers (Brand et al., 1976), while they are tens of microns apart in the UBC axons. The Eps8 immunoreaction product is present both within the constricted portions of the parallel fibers, which contain mainly cytoskeletal elements, and within the parallel fiber varicosities, which contain predominantly synaptic vesicles. Conversely, the core of the vesicle-rich mossy terminals of the UBC axon is distinctly Eps8-labeled, while the preterminal portion of the leading fiber is at best faintly stained. Taken together, these data point to a more prominent role for Eps8 in the axon terminals compared to the fibers.

Postsynaptically, our immunolocalization data suggest that Eps8 is closely associated with the postsynaptic apparatus. Immunostaining of the PSDs was evident in both granule cell and UBC dendrites. However, because labeling of the PSD by DAB reaction product might be due diffusion from antibody binding sites in the vicinal postsynaptic cytoplasm, we cannot ascertain to what extent, if at all, Eps8 co-localizes with the PSD. Resolving this issue awaits quantitative studies with postembedding immunogold, which detects antigens more precisely (Matsubara et al., 1996). Nonetheless, the association between Eps8 and the PSD is supported by our unpublished results indicating that Eps8, PSD95, Chapsyn 110/PSD93 and N-methyl-D-aspartate (NMDA) glutamate receptor subunit NR1 are released in the same membrane fractions after sodium dodecyl sulfate/deoxycholate, but not Triton-X 100, detergent extraction (see also Offenhauser et al., 2006).

The intensity of immunostaining in UBC perikarya is slightly higher than in granule cells, but this could merely reflect the different cell body sizes (9–10 μm versus 6–7 μm). The difference in immunostaining density in the dendritic termini of these cell types, however, is more apparent than that of their parent cell bodies and is paralleled by a difference in phalloidin binding. Within UBC dendritic brushes, we observed foci of Eps8-immunostaining distinctly greater than in any other UBC or granule cell compartment. Indeed we refer to these Eps8-labeled structures as “hotspots”, a term that accurately describes regions of intense Eps8 immunoreactivity surrounded by a larger area with lower Eps8 protein expression. That Eps8 hotspots are distinctly coincident with phalloidin hotspots suggests that Eps8 and actin microfilaments most likely interact within these different compartments.

While this manuscript was under revision, another group of investigators published a paper dealing with Eps8 expression by granule cells in vivo and in vitro in relational to ethanol toxicity, but without addressing the Eps8 labeling in UBCs (Offenhauser et al., 2006). Some of their results support our findings, namely: Eps8 protein is abundantly expressed in the mouse cerebellum, colocalizes with phalloidin in the granular layer, is expressed mostly in dendritic articulations surrounding mossy fibers, and is present in both the presynaptic synaptosomal/synaptophysin-positive and the postsynaptic density/PSD95-positive subcellular fractions.

Because Eps8, by associating with different binding partners, coordinates two important GTPase-mediated functions - actin reorganization and receptor internalization (Lanzetti et al., 2000; reviewed in Di Fiore and Scita, 2002)-, the colocalization of actin and Eps8 in different neuronal compartments raises an interesting question regarding its main function: Does Eps8 primarily regulate actin reorganization, or does it, by forming a scaffolding complex with actin, function primarily as a transducing molecule whose effects on actin reorganization are indirect? The results of Offenhauser et al (2006) demonstrating that ethanol-induced Eps8-dependent neuronal reorganization of F-actin is not mediated by direct Eps8/F-actin interactions, suggests that Eps8 regulation of actin dynamics is indirect, perhaps through other actin binding proteins, such as cofilin. Similarly, Ryan and colleagues (Sankaranarayanan et al., 2003) showed that the main function of actin in presynaptic boutons is to manage the availability of regulatory molecules, such as synapsin, rather than to control synaptic vesicle recycling directly. Taken

together, these results indicate that the primary role of actin/Eps8 scaffolding complexes in post- and presynaptic structures might be more dependent upon the diversity of available binding partners within different compartments of the same neuron.

If the primary function of actin in neurons is to corral signaling molecules, such as Eps8, then it follows that the spatial distribution of actin and its complement actin-binding proteins would be dynamic and dictated primarily by neuronal phenotypes. Such a phenomenon would explain the distinct differences in Eps8 staining intensity and distribution seen in granule cell and UBC dendrites, and the absence of Eps8 in other actin-rich structures. Indeed, the absence of Eps8 immunoreaction product both in the actin-rich Purkinje cell spines (Matus, 2005) and the basket axons of the pinceau (Capani et al., 2001a,b) indicate that actin interactions in cerebellum are regulated in a neuron class-specific manner. The absence of Eps8 in Purkinje cell spines, which are known to contain complexes formed by espin, an actin binding protein, and the insulin receptor tyrosine kinase substrate p53 (IRSp53) (Sekerková et al., 2003), is particularly interesting, given that IRSp53 in other cells can interact with Eps8 to regulate actin-mediated cell motility (Funato et al., 2004). That different cell types – including neurons and glial cells - regulate actin-mediated processes differently are further underscored by the exclusive localization of espins in Purkinje cell spines (Sekerková et al., 2003), drebrin and spinophilin in spines of hippocampal pyramidal neurons (reviewed by Ethell and Pasquale, 2005), and the ERM proteins ezrin and radixin in astrocytic lamellae (Tsukita and Yonemura, 1999;Derouiche and Frotscher, 2001).

Eps8 and synaptic remodeling

As previously demonstrated, the synaptic junctions at the mossy fiber-UBC contact occur on the shafts of the brush dendrioles in a variety of forms, ranging from elongated, individual or serially arranged junctions, to clusters of macular synapses (Mugnaini et al., 1994). In confocal immunofluorescence images, the variety of synaptic forms was reflected by the occurrence of macular and ellipsoid Eps8 hotspots, which represented en face or side-views of the specialized postsynaptic sites. That larger and smaller Eps8 hotspots occurred in both calretinin-positive UBCs and mGluR1α-positive UBCs indicated that the variety of synaptic junctions does not depend on the UBC chemical phenotypes.

By binding actin and acting as a scaffold supporting the formation of multiprotein signaling complexes that promote the activation of Rho family GTPases, Eps8 at UBC synapses is likely to participate in actin dynamics related to synaptic activity and turnover, in a manner analogous to that in dendritic spines. At the spines, actin 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 (Okamoto et al., 2004;Ethell and Pasquale, 2005;Nicholson-Dykstra et al., 2005). Eps8 is also thought to transduce extracellular signals in different cellular systems through its association with proteins that regulate actin remodeling and/or receptor endocytosis (Di Fiore and Scita, 2002;Martinu et al., 2002;Croce et al., 2004;Disanza et al., 2004,2005;Funato et al., 2004;Leu et al., 2004;Offenhauser et al., 2004;Roffers-Agarwal et al., 2005;Goicoechea et al., 2006;Khanday et al., 2006).

The main input to UBCs in the caudal cerebellum is provided by vestibular mossy fibers (Diño et al., 2000a,2001), and these are able to fire at unusually high rates (Lysakowski and Goldberg, 2004). By stimulating or silencing the extrinsic mossy fibers impinging upon UBCs in a given lobule, such as the vestibulo-cerebellar nodule, it might be possible to test the hypothesis that the long and segmented synaptic junctions at the mossy fiber-UBC appositions constitute the extremes of a continuum, the momentary appearance of which depends on the dynamic state of the interneuronal contact.

These synapses might undergo conspicuous plastic changes that are discernible in the light microscope. Specifically, the frequency of occurrence of the large Eps8 hotspots in UBC brushes should decrease if sustained vestibular stimulation were to cause fragmentation of the large synapses; the opposite would be expected if activity caused fragmented synapses to fuse into larger junctions.

Thus, the cerebellum offers two tractable model systems to study the specific role of Eps8 and related protein complexes in actin remodeling and in other complex activity-dependent signaling pathways, including receptor endocytosis (Tocchetti et al., 2003;Croce et al., 2004;Disanza et al., 2004,2005;Higgs, 2004;Ethell and Pasquale, 2005;Roffers-Agarwal et al., 2005): the granule cells, which are ubiquitous *in situ* and form an extensive synaptic network of neurites *in vitro* (Gallo et al., 1987;Offenhauser et al., 2006); and the UBCs, which have a restricted distribution *in situ*, but can be studied in fresh slices (Rossi et al., 1995) and long-term organotypic cultures (Nunzi et al., 2001;Russo et al., 2006) and offer the advantage of a giant brush synapse provided with Eps8 foci in register with an organized postsynaptic actin cytoskeleton.

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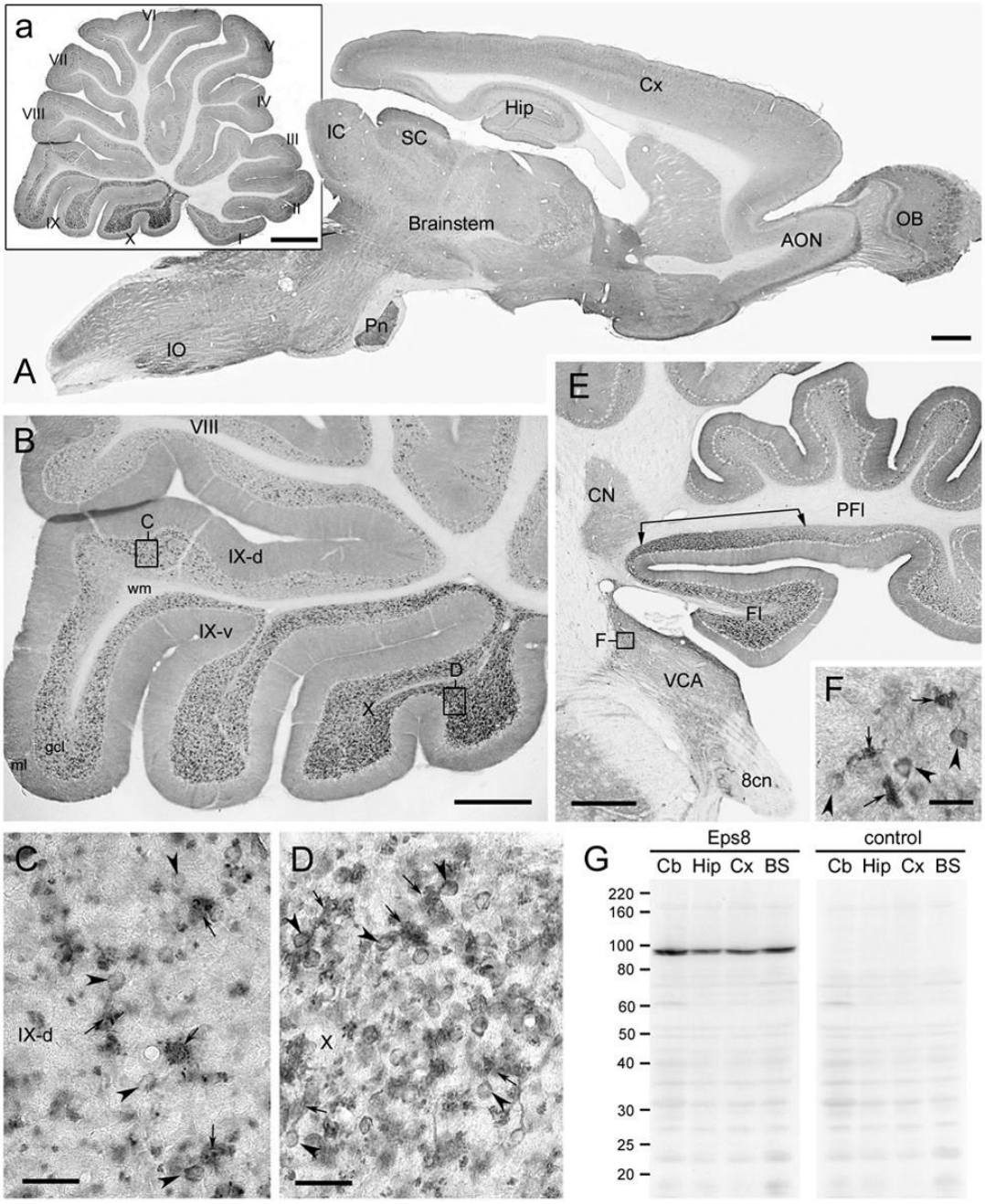

Fig 1.

Rat brain map of Eps8 by bright-field immunohistochemistry (panels A–F) and Western blots (panel G). (A) This sagittal section of the rat brain, with the cerebellum slightly enlarged as an inset (a), shows Eps8 immunoreactivity in various regions including the granular layer of cerebellar lobules IX and X (vestibulo-cerebellum). AON, anterior olfactory nuclei; Cx, cortex; Hip, hippocampus; OB, olfactory bulb. (B) Detail from a sagittal cerebellar section shows variable densities of immunostained structures in the granular layer (gcl); low in lobule VIII and dorsal uvula (IX-d), medium in ventral uvula (IX-v), and high in nodulus (X). Boxed areas are shown at higher magnification in panels C and D. Faint immunostaining in the molecular layer (ml) is attributed to parallel fibers, as Purkinje cells, stellate cells and basket cells are

clearly immunonegative; Wm, white matter. (C, D) Eps8 immunoreactivity in the cerebellar granular layer is localized to moderately stained cell bodies (arrowheads) and dendritic trunks and intensely stained neuropil areas identified as UBC cell bodies and dendritic brushes (arrows), respectively. Individual UBCs are clearly apparent in a folium with low densities of immunostained structures (panel C) and can also be identified in the intensely stained folium of lobule X (panel D). (E) Detail of the cerebellum and the adjacent brain stem in a coronal section shows high density of immunostained structures in the granular layer of the UBC-rich flocculus (Fl) and the ventral paraflocculus (line with two arrows, PFl). CN, lateral cerebellar nucleus; IC, inferior colliculus; IO, inferior olive; Pn, pontine nuclei; SC, superior colliculus; VCA; ventral cochlear nucleus; 8cn; nerve root of the 8^{th} nerve. (F) Eps8 immunopositive UBCs in the cochlear nucleus from the enlarged boxed area shown in E. Arrowheads depict moderately stained UBC cell bodies, while the arrows point to the intensely stained UBC dendritic brushes. (G) Western blots of cerebellum (Cb), hippocampus (Hip), cortex (Cx), and brain stem (BS) stained with (left) and without (right) Eps8 antiserum. Scale bars = 1 μm (A), 500 μm (a, B, E), and 20 μm (C, D, F).

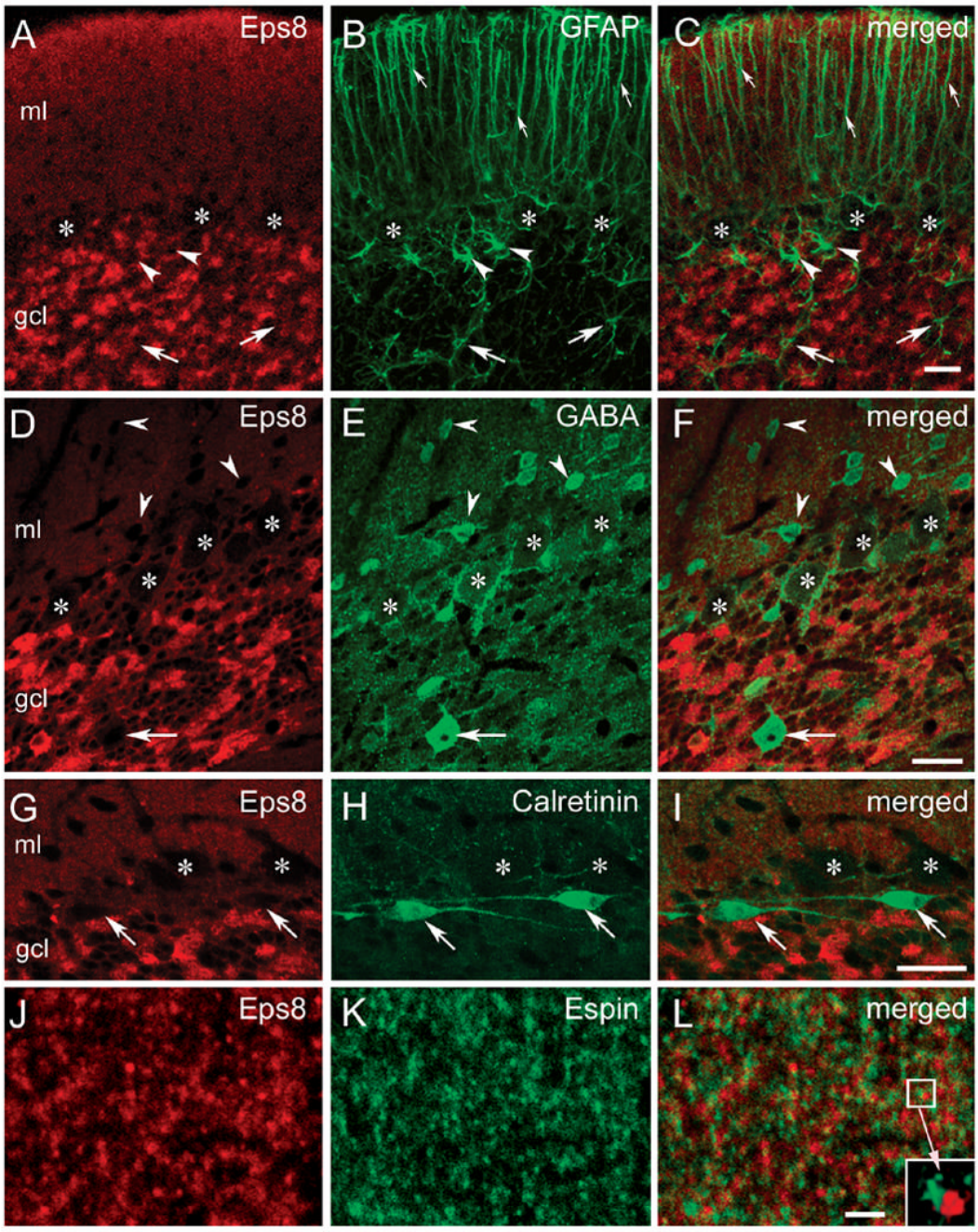

Fig 2.

Confocal images of cerebellar sections double-labeled with antibodies to Eps8 and a cell type specific marker. Eps8 immunoreactivity is absent in astroglia and inhibitory interneurons. (A–C) GFAP-positive Bergmann glia (arrowheads) and astrocytes (arrows) in the granular layer (gcl) and white matter are Eps8-negative. Eps8- and GFAP-negative Purkinje cells are labeled by asterisks. Small arrows depict Bergmann glial processes in the molecular layer. (D–F) GABA-positive Purkinje cells (asterisks), basket and stellate cells (arrowheads), and Golgi cells (arrow) are Eps8-negative. (G–I) Bipolar, calretinin-positive Lugaro cells, which are typically situated beneath the Purkinje cell layer, are also Eps8-negative. (J–L) Double labeling of the cerebellar molecular layer with antibodies to Eps8 and espin show no overlap between

Eps8-positive dots and the espin-positive Purkinje cell spines. In panel L an enlarged and processed image of the boxed area is shown as a small inset. A presumptive granule cell varicosity (red) is in close apposition to a presumptive Purkinje cell spine (green). Scale bars = 25 μm (A–I) and 2 μm (J–L).

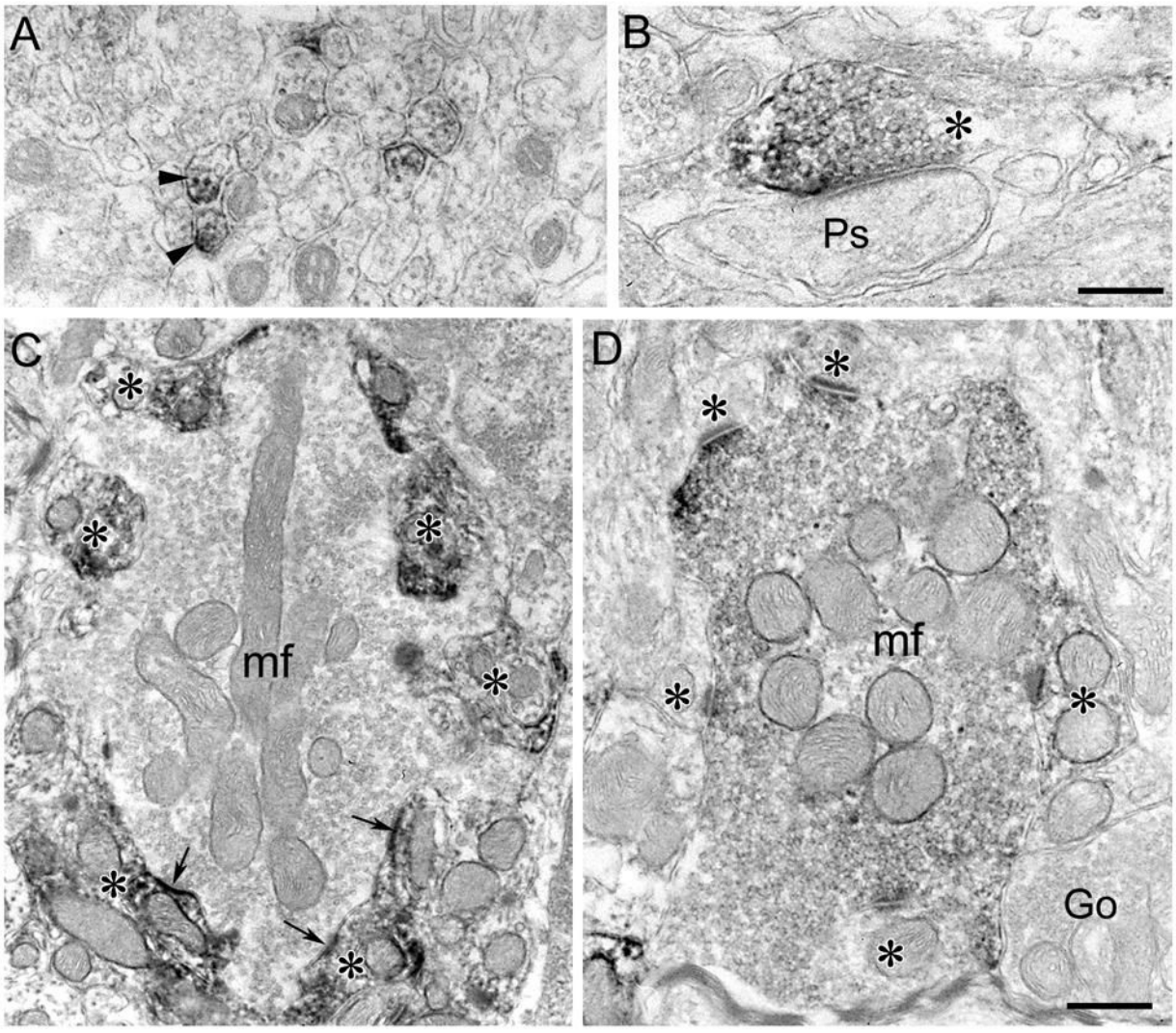

Fig 3.

Pre- and postsynaptic Eps8 immunoreactivity in granule neurons. Immuno-electron micrographs from the molecular (panels A and B) and granular (panels C and D) layers demonstrate Eps8 immunostaining in parallel fibers and cerebellar glomeruli. (A) Immunostained parallel fibers (arrowheads) in cross-section, from a slice cut transversely to the course of the folium. (B) Immunostained parallel fiber varicosity (asterisk) in contact with an immunonegative Purkinje cell spine (Ps), from a slice cut parallel to the course of the folium. (C) Immunostained granule cell dendrites (asterisks) surrounding an immunonegative mossy fiber terminal (mf, filled with mitochondria and small, clear synaptic vesicles) within a glomerulus; arrows indicate electron dense PSDs in granule cell dendrites. (D) Immunostained mossy fiber terminal (mf) within a glomerulus; Asterisks mark granule cell dendrites and Go indicates a Golgi axon terminal containing pleomorphic vesicles, characteristic of inhibitory neurotransmission. Scale bars = 2 μm (A), 0.2 μm (B), and 0.5 μm (C, D).

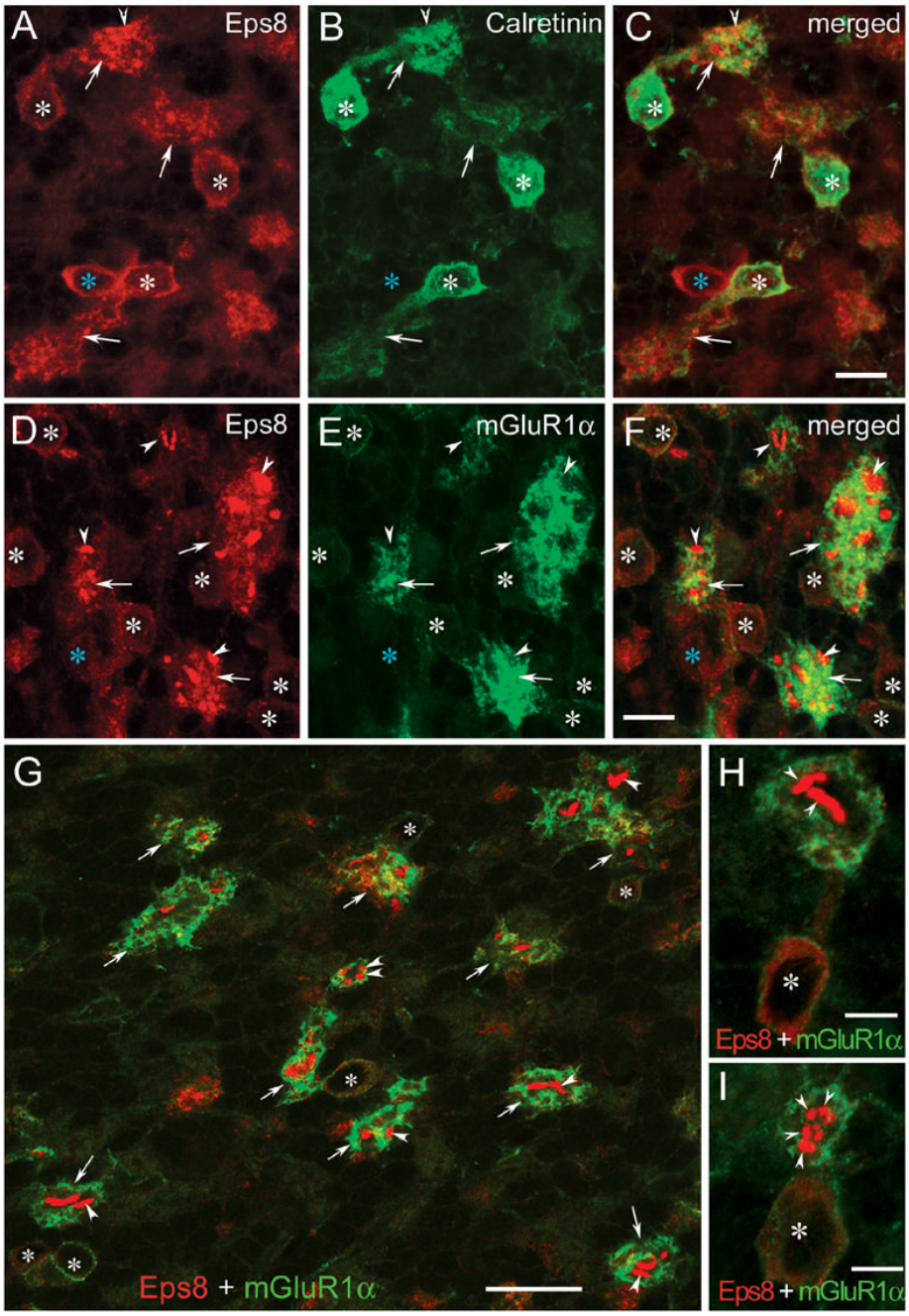

Fig 4.

Eps8 is present at high density in brushes of the two subsets of UBCs. Two-color confocal immunofluorescence microscopy demonstrates Eps8 (red) immunoreactivity in UBCs characterized by differential expression of calretinin (green; panels A–C) and mGluR1α (green; panels D–I). Panels G–I highlight the subcellular immuno-localization of Eps8 in mGluR1α-positive UBCs. Eps8 immunoreactivity is mostly localized to hotspots in the postsynaptic brush area, while mGluR1α immunoreactivity is strongest at the brush periphery. The Eps8 hotspots vary is number, shape and size in different brushes. (H) UBC with two elongated, Eps8 hotspots in the brush center. (I) UBC with numerous, patch-like Eps8 hotspots in the brush center. White asterisks indicate UBC cell bodies double-labeled with Eps8 and calretinin, or Eps8 and

mGluR1α; blue asterisks mark UBC cell bodies single-labeled with Eps8. Arrows point to UBC brushes, while the arrowheads point to Eps8 immunoreactive hotspots in the brush region. Scale bar = 5 μm (A–F), 20 μm (G) and 5 μm (H, I).

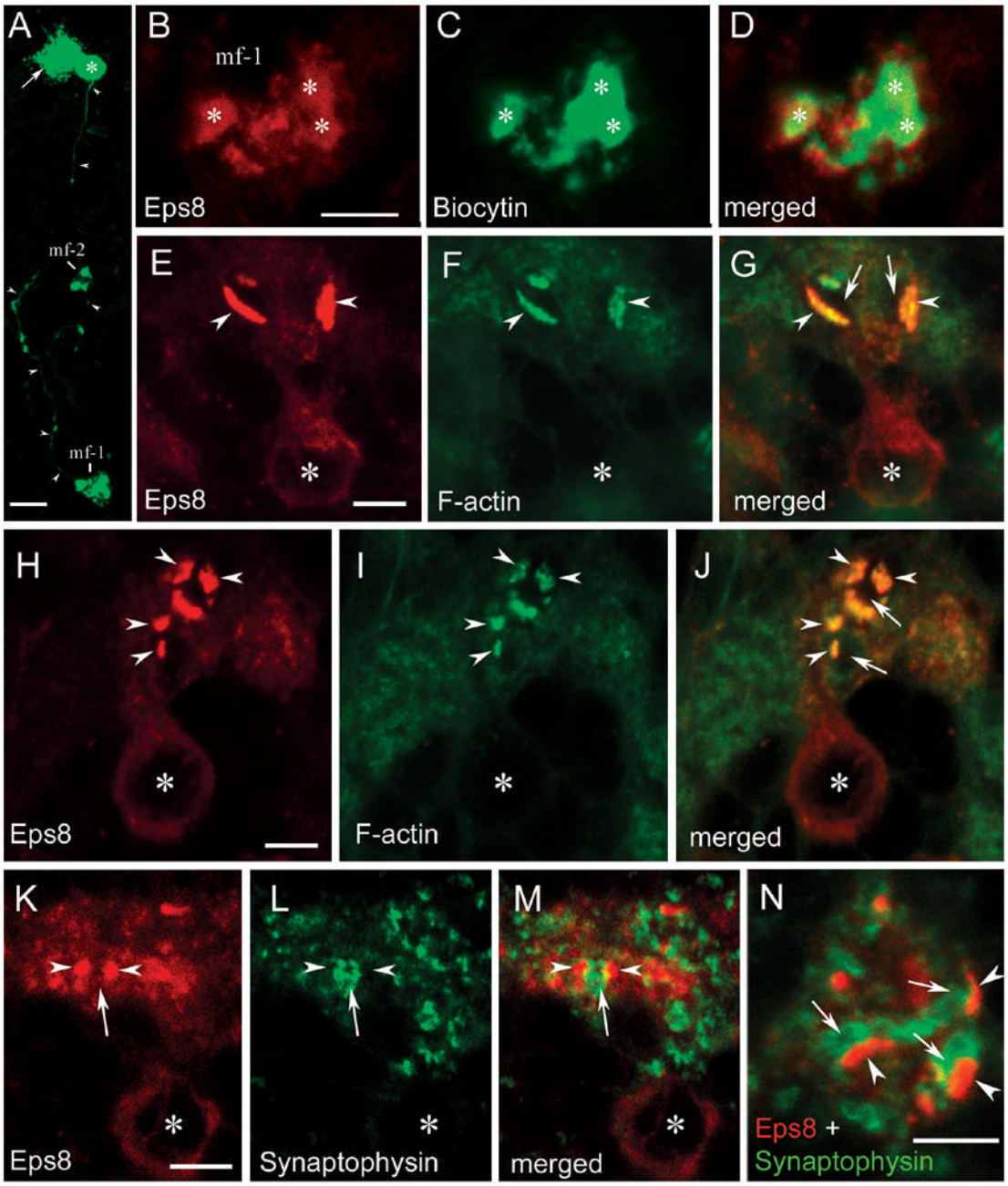

Fig 5.

Eps8 immunoreactivity is localized at low density in the UBC axonal terminals (A–D), but is present at very high density in the actin-rich postsynaptic region of the dendritic brush (E–N). Panel A shows a biocytin-filled UBC (arrow indicates the brush and asterisk the cell body). The labeled axon (arrowheads) forms two mossy terminals (mf-1 and mf-2). Panels B–D show the terminal labeled mf-1 in panel A, double-stained with biocytin and Eps8 antibody; asterisks in the main body of the mossy terminal indicate areas of distinct colocalization. Panels E–J highlight the colocalization of larger (E–G) and smaller (H–J) Eps8 (red) hotspots (foci) and phalloidin binding sites (green). Asterisks indicate UBC cell bodies and the arrowheads point to Eps8/F-actin enriched hotspots. Arrow in panels G and J point to unstained regions of the

brush area that were most likely occupied by the presynaptic mossy fiber. Panels K–N demonstrate the spatial relationship between Eps8 (red) hotspots (arrowheads) and the presynaptic terminals (arrow) labeled with vesicle marker synaptophysin (green). In panels K–M, binding sites (arrows) of antibodies to the synaptic vesicle marker synaptophysin (green) occupy the space between two large Eps8 (red) hotspots (arrowheads). Asterisk indicates the UBC cell body. In panel N, side-by-side localization of elongated (indicated by arrows and arrowheads) and macular (not indicated) Eps8 hotspots (red) and phalloidin (green) binding sites in the same UBC brush. Scale bar = 10 μm (A) and 5 μm (B–N).

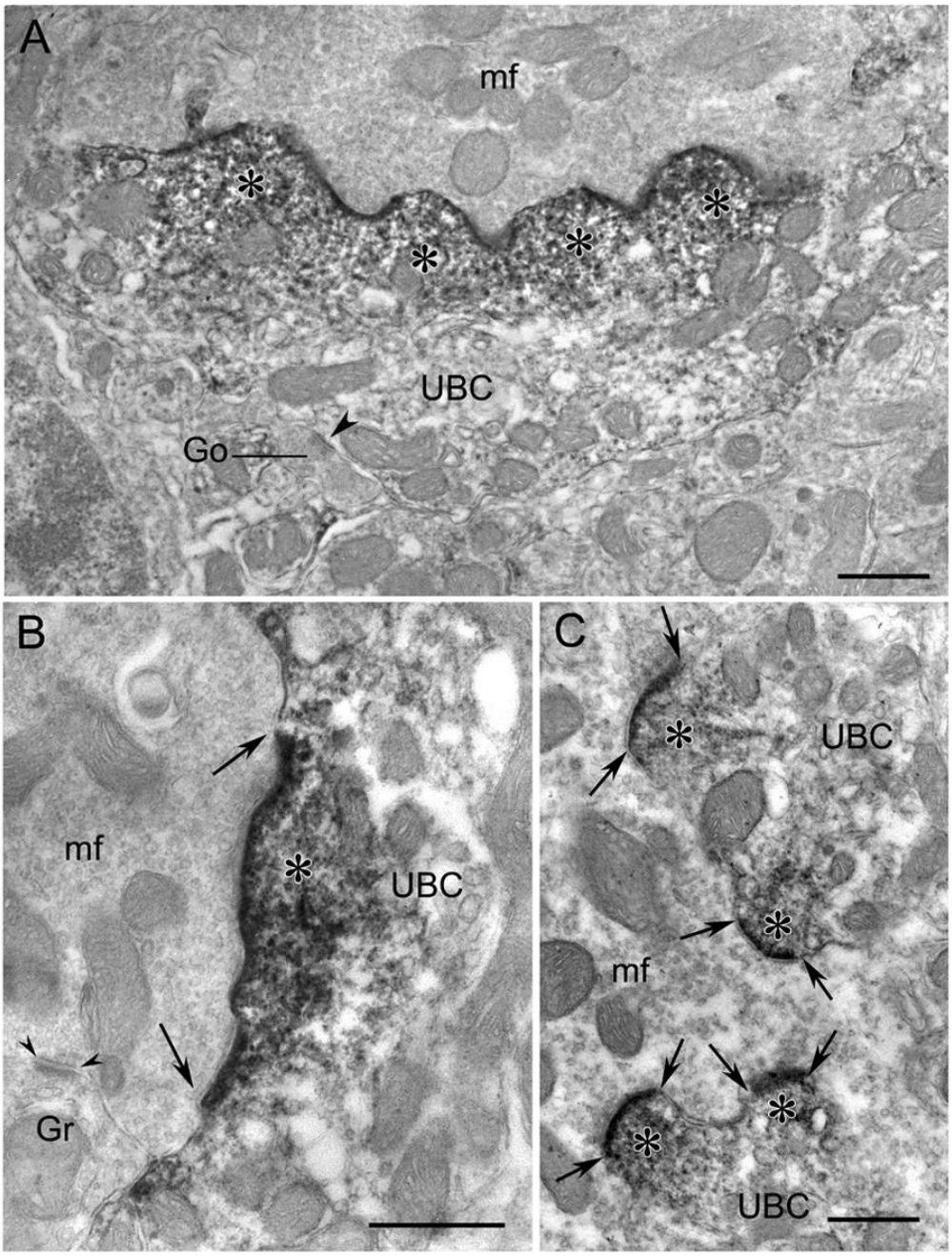

Fig 6.

Eps8 co-distributes with postsynaptic actin filaments at the mossy fiber-UBC synapse. Immuno-electron microscopy demonstrates DAB-immunoreaction product of Eps8 at the actin filament rich postsynaptic web and the postsynaptic densities of UBC dendrioles. (A) Scalloped, immunoreactive region of a UBC dendriole synaptically apposed to a mossy fiber (mf) ending. Go indicates a Golgi axon terminal forming a small, unlabeled, symmetric synapse (arrowhead) with the opposite site of a UBC dendriole. The Golgi terminal contains pleomorphic synaptic vesicles, characteristic of inhibitory neurotransmission. (B) This mossy fiber ending (mf) forms a large, uninterrupted synapse (between arrows) with a UBC dendriole; the postsynaptic web and the postsynaptic density are densely marked by Eps8/DAB

immunoreaction product. Gr indicates a granule cell dendrite forming a small, asymmetric synapse with the mossy ending. (C) This mossy fiber ending (mf) forms a synaptic contact with a UBC dendriole that is subdivided into four postsynaptic densities (between arrows), each provided with a separate Eps8 immunostained postsynaptic compartment. Scale bar = 0.5 μm.