Distinct subsynaptic localization of type 1 metabotropic glutamate receptors at glutamatergic and GABAergic synapses in the rodent cerebellar cortex

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Abstract

Type 1 metabotropic glutamate (mGlu1) receptors play a pivotal role in different forms of synaptic plasticity in the cerebellar cortex, e.g. long-term depression at glutamatergic synapses and rebound potentiation at GABAergic synapses. These various forms of plasticity might depend on the subsynaptic arrangement of the receptor in Purkinje cells that can be regulated by protein-protein interactions. This study investigated, by means of the freeze-fracture replica immunogold labelling method, the subsynaptic localization of mGlu1 receptors in the rodent cerebellum and whether Homer proteins regulate their subsynaptic distribution. We observed a widespread extrasynaptic localization of mGlu1 receptors and confirmed their peri-synaptic enrichment at glutamatergic synapses. Conversely, we detected mGlu1 receptors within the main body of GABAergic synapses onto Purkinje cell dendrites. Although Homer proteins are known to interact with the mGlu1 receptor C-terminus, we could not detect Homer3, the most abundant Homer protein in the cerebellar cortex, at GABAergic synapses by pre-embedding and post-embedding immunoelectron microscopy. We then hypothesized a critical role for Homer proteins in the peri-junctional localization of mGlu1 receptors at glutamatergic synapses. To disrupt Homer-associated protein complexes, mice were tail-vein injected with the membrane-permeable dominant-negative TAT-Homer1a. Freeze-fracture replica immunogold labelling analysis showed no significant alteration in the mGlu1 receptor distribution pattern at parallel fibre-Purkinje cell synapses, suggesting that other scaffolding proteins are involved in the peri-synaptic confinement. The identification of interactors that regulate the subsynaptic localization of the mGlu1 receptor at neurochemically distinct synapses may offer new insight into its trafficking and intracellular signalling.

Introduction

The cerebellum plays a key role in the coordination of movements as well as in the regulation of sensory, emotional and cognitive functions (Ito, 2001, 2006). Recent evidence suggests that multiple forms of plasticity regulate the acquisition, dynamics and consolidation of these learned behaviours (Boyden et al., 2004; Wang et al., 2014). The computations required to accomplish these different forms of plasticity occur in the topographically organized excitatory and inhibitory inputs to dendritic spines and shafts, respectively, of Purkinje cells (PCs), the sole output neurons of the cerebellar cortex, which are in turn controlled by several distinct interneurons (Raymond et al., 1996).

The PCs abundantly express in their spines and dendrites the type 1 metabotropic glutamate (mGlu1) receptor, which exists as multiple splice variants (Ferraguti et al., 2008). All mGlu1 isoforms are expressed by PCs including mGlu1a (Ferraguti et al., 2008), which possesses the longest C-terminus tail, enabling it to directly interact with multiple intracellular partners such as the Homer family of scaffolding proteins (Xiao et al., 2000). In PCs, mGlu1 receptors critically contribute to synaptic long-term depression after synchronous and repeated firing of parallel fibres (PFs) and climbing fibres (Aiba et al., 1994; Conquet et al., 1994; Shigemoto et al., 1994; Dzubay & Otis, 2002). In addition to long-term depression, mGlu1 receptors have been implicated in a number of other important activity-dependent synaptic changes at PC glutamatergic synapses, such as depolarization-induced suppression of excitation (Maejima et al., 2001). Plastic changes also occurring at GABAergic synapses were
shown to depend on mGlu1 receptors. These include depolarization-induced suppression of inhibition (DSI) (Galante & Diana, 2004) and rebound potentiation (RP) (Sugiyama et al., 2008).

The way in which mGlu1 receptors influence these various forms of plasticity probably arises from their precise localization and arrangement at synapses, as well as from their interaction with different scaffolding and signalling proteins (Ferraguti et al., 2008). Post-embedding immunogold labelling showed a peri-synaptic accumulation of mGlu1 receptors in an annulus surrounding the post-synaptic density (PSD) of both types of PF-PC and climbing fibre-PC excitatory inputs (Baude et al., 1993; Nusser et al., 1994; Lujan et al., 1997; Mateos et al., 2000), indicating a direct participation in the molecular plasticity mechanisms at these synapses (Kano et al., 2008). However, a spatial association between mGlu1 receptors and GABAergic synapses in the cerebellar cortex remains unexplored. Previous work in the basal ganglia suggested that mGlu1 receptors could reside in the main body of type II symmetric synapses (Hanson & Smith, 1999). However, because of the limitations of the pre-embedding immunolabelling method for the detection of intrasynaptic proteins, conclusive evidence for the presence of mGlu1 receptors in GABAergic synapses awaits the use of more sensitive approaches. With this aim in mind, we have exploited the freeze-fracture replica immunogold labelling (FRIL) method, which allows the visualization of integral membrane proteins with high spatial resolution and high sensitivity (Masugi-Tokita & Shigemoto, 2007). In addition, to address the mechanisms underlying the characteristic per-junctional localization of mGlu1 receptors in glutamatergic synapses, we have investigated the role of the long Homer proteins by intravenous injection of the dominant-negative Homer1a fused with the cell-penetrating peptide TAT.

Materials and methods

Ethical standards

Experimental procedures on animals were approved by the Austrian Animal Experimentation Ethics Board (GZ66.011/83-BrGT/2005 and GZ66.011/28-BrGT/2009) and the National Institute for Physiological Science’s Animal Care and Use Committee, and were in accordance with directives of the French Ministry of Agriculture, in compliance with both the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used, and to use alternatives to in vivo techniques whenever available.

Experimental animals

Both adult male rats and mice were used for this study. Sprague Dawley rats (300–400 g) were obtained from the Department of Laboratory Animals and Genetics (n = 5; Medical University Vienna, Vienna, Austria) and Japan SLC Inc. (n = 3; Hamamatsu, Japan). Adult male C57Bl/6 mice (n = 11; 25–30 g; Charles River, Sulzfeld, Germany) as well as mGlu1 knockout (KO) mice (n = 5; backcrossed in C57Bl/6 for five generations) and wild-type littermate mice (n = 5; bred at the Department of Pharmacology, Innsbruck, Austria) were also used. Injection of TAT-Homer1a or TAT-HomerW24Y was carried out in adult male Swiss mice (n = 14; 8 weeks old; Janvier, St Berthevin, France). Before use, the animals were housed in groups of four to five under controlled laboratory conditions (12:12 h light/dark cycle with lights on at 07:00 h; 21 ± 1 °C; 60% humidity) with food and water ad libitum for at least 2 weeks after delivery from the supplier. Animals were deeply anesthetized by intraperitoneal injection of thiopental (150 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS) (0.9% NaCl, pH 7.4) followed by ice-cold fixative.

Suppliers

Thiopental was obtained from Sandoz (Kundl, Austria). Normal goat serum, biotinylated antibodies and the avidin-biotinylated horseradish peroxidase complex were purchased from Vector Laboratories (Burlingame, USA). Fab fragments and antibodies coupled to nanogold particles as well as the HQ silver enhancement kit were obtained from Nanoprobes (Stony Brook, NY, USA). Paraformaldehyde, osmium tetroxide and uranyl acetate were from Agar Scientific Ltd (Stansted, UK). Electron microscopic grade glutaraldehyde was purchased from Polysciences Inc. (Warrington, PA, USA) and picric acid from Fluka GmbH (Buchs, Switzerland). Dynabeads Protein-A, Seelblue Plus2 Prestained Protein Marker, NuPAGE 4–12% Bis-Tris gel and 3-(N-Morpholino)propanesulfonic acid sodium dodecyl sulfate Running Buffer were all obtained from Invitrogen. The polyvinylidene difluoride membrane and ECL Prime were from GE Healthcare. All other chemicals were purchased from Sigma (Vienna, Austria).

Reagents

The source of the antibodies, antigen used, specificity and dilutions are given in Table 1. Whenever possible, the specificity of primary antibodies was tested in tissue from KO animals. To control for possible cross-reactivity between IgGs in double-immunolabelling experiments, some sections were processed through the same immunocytochemical sequence except that only one primary antibody was applied, but the full complement of secondary antibodies was maintained. All of these control reactions resulted in a lack of labelling of the species-unrelated secondary antibodies, further confirming the specificity of the immunosignals.

Immunocytochemistry for light and pre-embedding electron microscopy

For these experiments animals were perfused with a fixative made of 4% w/v paraformaldehyde and 15% v/v of a saturated solution of picric acid in phosphate buffer (PB) (0.1 M, pH 7.4) for 15 min for rats or 10 min for mice. For electron microscopy experiments glutaraldehyde (25%) at a final dilution of 0.05% v/v was added to the fixative just before the perfusion. Brains were then immediately removed from the skull, washed in 0.1 M PB and sliced coronally in 40-μm-thick (for light microscopy) or 70-μm-thick (for electron microscopy) sections on a vibratome (VT1000S, Leica Microsystems, Vienna, Austria). Sections were stored in 0.1 M PB containing 0.05% sodium azide at 6 °C until use. Pre-embedding immunocytochemistry experiments were carried out according to previously published procedures (Sreepathi & Ferraguti, 2012). Briefly, free-floating sections were freeze–thawed twice after cryoprotection in 20% sucrose in 0.1 M PB to allow antibody penetration, and then incubated in 20% normal goat serum in Tris-buffered saline (TBS) for 2 h at 21 °C–23 °C (room temperature, RT). After blocking, sections were exposed first to primary antibodies (see Table 1) for ~72 h at 6 °C and then with the appropriate secondary antibodies (Table 1) overnight at 6 °C, both made up in a solution containing 2% normal goat serum in TBS. For light microscopy the immunore-
action was visualized by means of horseradish peroxidase and 3'-diaminobenzidine (0.5 mg/mL). The sections were then mounted onto gelatin-coated glass slides, dehydrated (50%, 70%, 90%, 95%, 100% and butylacetate) and finally coverslipped with Eukitt (Agar Scientifíc Ltd). Analysis was performed under an AxiosPhot microscope (Zeiss, Jena, Germany). Images were taken through an Axios-Cam camera (Zeiss) by means of the OPENLAB software (version 5.5.0; Improvision, Coventry, UK).

For electron microscopy the immunoreaction was visualized by means of a nanogold–silver-enhanced reaction. Sections were incubated overnight with Fab’ fragment secondary antibodies conjugated to nanogold particles (1.4 nm), then extensively washed in milliQ water followed by silver enhancement of the gold particles using the HQ kit (NanoProbes) for ~10–12 min. After extensive washes in milliQ water and 0.1% PB, the sections were treated with 2% OsO4 in 0.1% PB for 40 min at RT. Contrast was enhanced with 1% uranyl acetate in 50% ethanol for 30 min at RT. Sections were dehydrated by graded ethanol (50%, 70%, 90%, 95%, 100%) and propylene oxide at RT, and then quickly transferred into weighing boats containing epoxy resin (Durcupan ACM-Fluka, Sigma) where they were kept overnight at RT. The following day, the sections were transferred onto siliconized slides, coverslipped with ACLAR® film coverslips (Ted Pella, Inc., Redding, CA, USA), and incubated for 3 days at 60 °C. Blocks of the cerebellar cortex were cut under a stereomicroscope and re-embedded in epoxy resin. Ultrathin sections (70 nm) were cut using a diamond knife (Diatome, Switzerland) on an ultramicrotome (Ultracut, Leica), collected on single-slot copper grids coated with pioloform (Agar Scientific Ltd) and analysed in a transmission electron microscope (CM120, Philips, Eindhoven, the Netherlands) equipped with a Morada CCD camera (Soft Imaging Systems, Münster, Germany).

**Post-embedding immunogold labelling**

For post-embedding experiments animals were perfused with a fixative solution containing 4% paraformaldehyde and 0.05% glutaraldehyde for 15 min, with no post-fixation. Tissue blocks were dissected from the cerebellar cortex, freeze-substituted and embedded at low temperature in Lowicryl HM20 resin. Ultrathin sections (70–80 nm) were cut on an ultramicrotome, mounted on formvar-coated nickel mesh grids, and processed for immunogold cytochemistry as previously described (Kaufmann et al., 2009). Sections were etched with 0.05% polyethylene glycol) for 90 min at RT. The sections were pre-blocked with primary antibodies (Table 1) diluted in TBS-T containing 2% bovine serum albumin overnight at 4 °C. After rinsing in TBS-T, gold-conjugated goat anti-rabbit secondary antibody was applied (diluted 1 : 30 in TBS-T containing 2% bovine serum albumin and 0.05% polyethylene glycol) for 90 min at RT. The sections were then rinsed in double-distilled water and air-dried. The sections were stained with uranyl acetate (4% uranyl acetate w/v in 50% ethanol; 1.5 min at RT) and 0.3% lead citrate (1.5 min at RT), and examined in a Philips CM120 transmission electron microscope.

TABLE 1. Concentrations and combinations of primary and secondary antibodies

| Primary antibody | Source and specificity | Antigen | Species | Dilution | Secondary antibody | Dilution | Combinations |
|------------------|------------------------|---------|---------|----------|--------------------|----------|--------------|
| mGlu1α C-t       | Shigemoto et al. (1994) | Rat aa 859-1199 | Rabbit | 1 : 1000 | BBI 4049, 10 nm, goat | 1 : 30 | – |
| Frontier Institute Co. Ltd, code no.: mGluR1α-Gp-Af660-1 | | Mouse aa 945-1127 | Guinea pig | 1 : 500 | BBI 4847, 5 nm, goat | 1 : 30 | A |
| Frontier Institute Co. Ltd, code no.: mGluR1α-Rb-Af811 | | Mouse aa 945-1127 | Rabbit | 1 : 2000 | Aurion, 6 nm, goat | 1 : 30 | B, C |
| mGlu1 N-t        | Ferraguti et al. (1998) | Rat aa 82-520 | Rabbit | 1 : 300 | BBI 4847, 5 nm, goat | 1 : 30 | G |
| Pan-AMPA         | Frontier Institute Co. Ltd, code no.: PanAMPAR-Gp-Af580-1 | Mouse aa 717-754 | Guinea pig | 1 : 200 | BBI 5913, 10 nm, goat | 1 : 30 | E |
| Masugi-Tokita & Shigemoto (2007) | | Mouse aa 520-533 | Guinea pig | 1 : 500 | BBI 5913, 10 nm, goat | 1 : 30 | F |
| Glu-62 C-t       | Frontier Institute Co. Ltd, code no.: GluRd2C-Rb-Af500-1 | Mouse aa 852-931 | Rabbit | 1 : 100 | BBI 6013, BBI 12701, 5 nm, goat | 1 : 30 | E |
| GABAα-γ (1)     | Kaufmann et al. (2009) | Mouse aa 328-382, GST fusion protein | Guinea pig | 1 : 100 | BBI 5913, 10 nm, goat | 1 : 30 | A |
| Kasugai et al. (2010) | | Rat aa 328-382, GST fusion protein | Rat | 1 : 200 | Aurion, 6 nm, goat | 1 : 30 | – |
| Homer3 (Vesl 3)  | Synaptic System, cat. no. 160 303 | Rat aa 122-177 | Rabbit | 0.8 µg/mL | BBI 3347, 15 nm, goat | 1 : 30 | C, G |
| Shiraishi et al. (2004) | | Mouse aa 131-358, EKQLS16EDL of human oncogene c-Myc | Rabbit | 1 : 3000 | Nanogold | 1 : 100 | Pre-embedding, LM Immunohistochemistry |
| Sigma, cat. no. M4439 | | EKQLS16EDL of human oncogene c-Myc | Mouse | 1 : 1000 | Anti-mouse Cy3-conjugated | 1 : 1000 | – |

Capital letters indicate combinations of antibodies used for FRIL reactions. aa, aminoisod acid; BBI, British Biocell International; C-t, carboxy terminus; Glu, glutamate; GST, glutathione S-transferase; LM, light microscopy; N-t, amino terminus.
TAT-Homer1a expression and purification

A DNA plasmid containing the open reading frame for Homer1a was encoded under the control of the cytomegalovirus promoter in pRK5-Homer1a, as previously described (Perroy et al., 2008). The Homer1a coding sequence was fused to the coding sequence of the TAT-permeant peptide to obtain pET-His-Myc-TAT-Homer1a. To engineer pET-His-Myc-TAT-Homer-W24Y, we used primers containing the point mutations coding for W24Y (5′-ACA-AAGAAGAACTATGCTCCCACTGTAAGCT-3′) to amplify by polymerase chain reaction the Homer1a coding sequence. BL21 (DE3)-competent cells were transformed with either the pET-His-Myc-TAT-Homer-W24Y vector or the pET-His-Myc-TAT-Homer vector and incubated in lysogeny broth media. Induction of protein synthesis was started by adding isopropyl-β-D-thiogalactopyranoside (500 μM) for 3 h. His-Myc-TAT-Homer1a protein was purified under denaturing conditions on Ni-nitrilotriacetic acid resin (Qiagen). Cells were lysed in 8 M urea buffer, pH 8, and centrifuged at 10,000 g for 20 min. Supernatants were loaded on Ni-nitrilotriacetic acid resin and incubated for 60 min to allow for protein binding. The resin was then washed in 8 M urea buffer, pH 6.5. The His-Myc-TAT-Homer1a protein obtained was as follows:

\[ \text{M}^\text{HHHHHRPGYGRKKRRQRRRGLD} \text{KLNL}^\text{SFDVDPEDQK} \text{L}^\text{IESEDLGGMGEQP}^\text{IPSTRAYHVQIDPN}^\text{KKNWVP}^\text{SKHAV} \text{V}^\text{SVFY}^\text{YDSTRN}^\text{VYRIISLDGSKAI}^\text{NSTITTP}^\text{NMTFTKTSQ}^\text{KFGQWA}^\text{DSRANTVYG}^\text{LGS}^\text{HSLKFS}^\text{AKFQEFK}^\text{EAARLAKEK}^\text{SQEK}^\text{MELTS}^\text{TSPQESAGDLQ}^\text{SLTPESING}^\text{TDERTPD}^\text{VTQNSEPRAP}^\text{EPTQNLAPPHRYTFNSAI}^\text{MIK} \]

The tryptophan amino acid modified in the W24Y mutant is highlighted in bold and underlined. The poly-His tag and Myc tag are also underlined. His-Myc-TAT-Homer1a was eluted by the addition of 8 M urea buffer, pH 4.5. Just before use, denatured His-Myc-TAT-Homer1a protein was desalted using a PD-10 column (GE Healthcare) and eluted in PBS.

In vivo TAT-Homer1a protein injection, tissue preparation and immunofluorescence

Swiss mice were intravenously injected with either a control saline solution (n = 3) or TAT-Homer1a (n = 3; 8 mg/kg each; injection volumes in the caudal vein: 100–150 μL). For immunofluorescence experiments, mice were anaesthetized at 60 min after injection and perfused transcardially with 4% paraformaldehyde in PBS followed by post-fixation for 48 h in the same solution. Brains were cut in sagittal sections (50 μm thick) with a vibratome, permeabilized for 3 h at RT in a solution containing 0.25% Triton, 10% goat and 10% donkey serum in PBS, incubated overnight at 4 °C with mouse anti-Myc antibody (Sigma; 1 : 1000 dilution), rinsed in PBS and incubated for 1 h at RT with an anti-mouse Cy3-conjugated antibody (Jackson Laboratory; 1 : 1000 dilution). Slices were mounted with Mowiol for observation under a Zeiss AxiosImager Z1 microscope equipped with Apotome and appropriate epifluorescence and filters (545 ± 25 and 605 ± 70 nm for excitation and emission, respectively). Image quantifications were determined with IMAGEJ software (National Institutes of Health, USA).

Fracture replica immunogold labelling method

Rats and mice were perfused transcardially for 7 min with a solution containing 0.5% or 1% paraformaldehyde and 15% of a saturated solution of picric acid in 0.1 M PB at a rate of 10 or 5 mL/min, respectively. TAT-Homer1a (n = 3) or TAT-Homer1a-W24Y (n = 3)-treated mice were perfused at 30 min after the intravenous injection. Brains were quickly extracted from the skull and sliced (150 μm thickness) on a vibratome (Leica). Slices were cryoprotected in 30% glycerol in 0.1 M PB and high-pressure frozen by means of an HPM 010 machine (Bal-Tec, Balzers, Liechtenstein). The frozen slices were then freeze-fractured at −115 °C and replicated with a first layer of carbon (5 nm), shadowed by platinum (2 nm), and followed by a second carbon layer (15 nm) in a freeze-etching BAF 060 device (Bal-Tec). After thawing, the tissue attached to replicas was solubilized with shaking at 80 °C overnight in the following solubilization solution: 15 mM Tris(hydroxymethyl)-aminoethane, 20% sucrose, and 2.5% sodium dodecyl sulphate, pH 8.3. Immunolabelling of replicas was carried out according to previously published procedures with minor modifications (Kaufmann et al., 2009). Blocking was performed with a solution consisting of 5% bovine serum albumin and 0.1% Tween-20 in TBS (pH 7.4). Replicas were incubated in primary antibodies (Table 1) at 15 °C for

![Fig. 1. Peri-synaptic localization of mGlu1α receptors at cerebellar PF–PC synapses.](image)
Swiss mice were injected intravenously with either TAT-Homer1a (n = 3) or TAT-Homer1aW24Y (n = 3); the cerebella were disected at 60 min after injection and snap frozen. The cerebella were pooled and homogenized in ice-cold 10 mM Tris-HCl, pH 7.4, buffer containing 320 mM sucrose, 1 mM phenylmethylsulphonyl fluoride, 1 mM NaF, 1 mM Na3VO4 and complete EDTA-free protease inhibitors (Roche, Vienna, Austria) using a motorized homogenizer (Sartorius). The P2 fractions were obtained by sequential centrifugation at 1000 g and 17 000 g. For immunoprecipitation, the detergent lysates were obtained by suspending the P2 fractions in ice-cold 25 mM Tris-HCl, pH 7.5, buffer containing 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 137 mM NaCl, 3 mM KCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM NaF, 1 mM Na3VO4 and complete EDTA-free protease inhibitors (Roche) followed by 60 min centrifugation at 20 000 g. Proteins (400 µg) were then incubated with 0.8 µg of mGlu1α receptor polyclonal antibody raised in guinea pig (Frontiers Institute, cat. no. Af660) for 2 h at 6 °C. The antigen–antibody complexes were then incubated with Dynabeads Protein-A (20 µL) and the immunoprecipitation eluates were obtained by heating the samples in Laemmli loading buffer for 10 min at 70 °C. The immunoprecipitation eluates were loaded on a pre-cast Nupage 4–12% Bis-Tris gel (Invitrogen) and then transferred to a polyvinylidene difluoride membrane that was cut below 97 kDa. The upper parts of the polyvinylidene difluoride membranes were immunolabelled with anti-mGlu1α antibodies (1 : 3000, rabbit polyclonal, Frontiers Institute, cat. no. Af811-1). The lower parts of the polyvinylidene difluoride membranes were first immunolabelled with anti-c-Myc antibodies (1 : 5000, mouse monoclonal, Sigma, cat. no. M4439) and, after stripping with β-mercaptoethanol (100 mM), reimmunolabelled with anti-Homer1 (1 : 1000, rabbit polyclonal, Frontiers Institute, cat. no. Af1000-1). Immunoreactive bands were detected by incubating the membranes in horseradish peroxidase-conjugated secondary antibodies (1 : 10 000, Invitrogen) followed by the ECL Prime reagent. Chemiluminescence was visualized with the Fusion SL-4 Vilber Lourmat imaging system (Peqlab, Erlangen, Germany).

Processing of image data

Whole images were contrast-adjusted, sharpened and cropped in Photoshop (Adobe) without changing any specific feature.

Sampling and analysis of gold particles

After immunogold labelling, to establish the distribution of mGlu1 receptors in relation to the synapse, defined as the intramembrane particle (IMP) cluster on the exoplasmic face, we measured the shortest distance from the centre of each 5 nm immunogold particle to the synaptic edge. Values inside the PSD were considered negative and those outside were considered positive. The particles that were present directly on the synaptic edge were given a value equal to zero. A synapse was considered positive for AMPA or Glu52 receptors when the PSD contained at least three gold particles. Results were then plotted using Prism-5 for Mac (GraphPad
Software, Inc.). The frequency of immunogold particles was measured in 60-nm-wide bins, keeping the edge of the synapse as 0. The distance data of gold particles from the synaptic edge obtained from different animals of the same species or treatment group were pooled. The density of immunogold particles detecting mGlu1α receptors in GABAergic synapses (total synaptic area analysed: 13.2 μm², measured from 131 synapses from three mice) and the extrasynaptic area (total synaptic area analysed: 241.0 μm²) on the protoplasmic face (P-face) of mouse PCs was measured from digital images at 53 000× magnification with IMAGEJ (version 1.45S).

Analysis of the frequency distributions of the gold particle distance between TAT-Homer1α-injected and TAT-Homer1αW24Y-injected mice was performed by means of the two-sample Kolmogorov–Smirnov test (SPSS Statistics software version 20, IBM). The density data for AMPA or Gluδ2 receptors were also pooled as there was no statistical difference between individual animals in the same group (Kruskal–Wallis test; AMPA, P = 0.5; Gluδ2, P = 0.1, control group). Data in text are given as mean ± SEM.

Results

**Peri-synaptic enrichment of metabotropic glutamate receptors type 1 at glutamatergic parallel fibre–Purkinje cell synapses**

Using the FRIL technique, we first re-examined the subcellular localization of the mGlu1α receptor isoform in the molecular layer of the rodent cerebellar cortex. Because the sequence specifying the mGlu1α receptor splice variant lies in the intracellular carboxy terminal tail, immunogold labelling was observed only on the P-face of replicas. In the cerebellar cortex, immunogold particles for mGlu1α receptors were observed throughout the PC soma, dendrites and spines including the spine neck. An apparent peri-synaptic enrichment of mGlu1α receptors was observed at PF–PC synapses (Fig. 1A). However, as the characteristic cluster of IMPs identifying glutamatergic synapses on the replica exoplasmic face cannot be seen on the P-face, we performed double-labelling experiments for mGlu1α and glutamate δ2 receptors. The glutamate δ2 receptor is an ionotropic receptor selectively present within the main body of PF–PC synapses; although it does not bind glutamate (Matsuda & Yuzaki, 2012) its gating is triggered by mGlu1α receptors (Ady et al., 2014). These experiments confirmed the presence of mGlu1α receptors at the edge of the PSD of PF–PC synapses (Fig. 1B,C). The specificity of the immunolabelling was confirmed on replicas obtained from mGlu1-KO mice (Fig. 1D,E). It is noteworthy that some IMP clusters present on the P-face of PC dendrites were also found to be densely labelled for mGlu1α receptors.
was further validated by double-labelling experiments for mGlu1 and receptor splice variants (Ferraguti raised against the extracellular N-terminus common to all mGlu1 the detection of mGlu1 receptors on the exoplasmic face, an antibody © 2014 The Authors.

Intrasynaptic localization of metabotropic glutamate receptors type 1 at GABAergic synapses

Following the original observation of Somogyi and co-workers (Kasugai et al., 2010) that, in replicas, GABAergic synapses can be visualized as clusters of IMPs on the P-face of hippocampal pyramidal cells, we also detected on the P-face of cerebellar PC dendrites IMP clusters strongly labelled for the GABA_A receptor subunits z1 (Fig. 5A) and β3 or neuroligin 2 (data not shown). To establish whether mGlu1z receptor-labelled IMP clusters on PC dendrites correspond to GABAergic synapses, we carried out double-labelling experiments for the GABA_A z1 subunit and mGlu1z receptor. Indeed, clear co-localization of the two molecules was detected within the main body of GABAergic synapses (Fig. 3B–D). To establish whether mGlu1 receptors are enriched in GABAergic synapses, we analysed the density of immunogold particles (5 nm) for mGlu1z receptors within GABAergic synapses (170.3 ± 13.8 gold particles/µm²), identified by sequential labelling for GABA_A z1 subunits, and in the nearby extrasynaptic areas (35.9 ± 3.5 gold particles/µm²). GABAergic synapses showed a significantly higher density (P < 0.0001; Mann–Whitney test) with a ratio of 7.8 ± 0.8.

To further corroborate the intrasynaptic localization of mGlu1z receptors in GABAergic synapses, we examined, in replicas of the mouse globus pallidus, the co-existence between GABA_A z1 subunits and mGlu1z receptors. Similar to what was observed in PC dendrites, IMP clusters onto the P-face of pallidal neuron dendrites also showed co-labelling for GABA_A and mGlu1z receptors (Fig. 4). Taken together, these findings demonstrated a differential subsynaptic localization of mGlu1 receptors in neurochemically distinct synapses and validated previous pre-embedding studies (Hanson & Smith, 1999; Smith et al., 2001).

Restricted localization of Homer3 to asymmetric synapses

The differential subsynaptic distribution of mGlu1 receptors between glutamatergic and GABAergic synapses raises the intriguing question of which molecular mechanisms are involved in the subcellular targeting of this receptor.

Homer proteins have been implicated in cross-linking group I mGlu receptors to inositol triphosphate receptors and phospholipase B4 (Brakeman et al., 1997; Nakamura et al., 2004). Several members of the Homer family have been identified (namely Homer1a, Homer1b-c, Homer2 and Homer3), which are characterized, with the exception of Homer1a, by a long coil-coiled carboxy terminal domain allowing multimerization (Xiao et al., 1998, 2000). Subcellularly, it has been suggested that long Homers and mGlu1z receptors co-localize at the periphery of the PSD of glutamatergic synapses (Xiao et al., 1998, 2000). Therefore, it can be hypothesized that long Homer proteins are involved in the specific peri-synaptic localization of mGlu1 receptor in glutamatergic synapses by restraining them from entering the post-synaptic specialization. The presence of mGlu1 receptors within GABAergic synapses would also be warranted by a lack of long Homer proteins in these synapses. In order to test this hypothesis, we used both pre-embedding and post-embedding techniques to reveal the subsynaptic distribution of the Homer3 protein, which is the most highly expressed long Homer isoform in the cerebellar cortex (Shiraishi et al., 2004). Our results showed the enrichment of Homer3 immunoreactivity in PCs (Fig. 5A) and demonstrated that Homer3 was indeed exclusively present at PSD boundary consistent with previous studies based on other techniques (Nusser et al., 1994; Lujan et al., 1997; Mateos et al., 2000).

Fig. 5. Lack of expression of Homer3 at symmetric synapses of PCs. (A) Light micrograph showing Homer3 expression in somata and dendrites of PCs as revealed by 3-3'-diaminobenzidine/horseradish peroxidase staining. Pre-embedding (B and C) and post-embedding (D and E) were used to determine the subcellular localization of Homer3 in asymmetric and symmetric synapses in the cerebellar cortex. (B and D) Immunogold particles for Homer3 are abundantly detected in asymmetric synapses of the dendrites of PCs. (C and E) Homer3 labelling was not detected in symmetric synapses. Ax t, axon terminal; den, dendrite; s, spine. Scale bars: 100 µm in A; 200 nm in B, D and E; 500 nm in C. The peri-synaptic enrichment of mGlu1 receptors at PF–PC synapses was further validated by double-labelling experiments for mGlu1 and δ2 receptors on both plasma membrane leaflets (Fig. 2A–D), using, for the detection of mGlu1 receptors on the exoplasmic face, an antibody raised against the extracellular N-terminus common to all mGlu1 receptor splice variants (Ferraguti et al., 1998). To establish the relative density of mGlu1 receptors in relation to the synapse, the distance of gold particles from the nearest PSD boundary was measured. Our data clearly demonstrated a skewed distribution toward the extrasynaptic area with a preferential concentration of gold particles (~50%) in an arbitrarily chosen 60 nm segment spanning the PSD edge (Fig. 2E). Taken together, our findings obtained with the FRIL method demonstrated that the density of mGlu1 receptors was highest at the edge of PF–PC synapses, dropping markedly as a function of distance from the
Homer1a-mediated disruption of long Homer complexes is not sufficient to alter metabotropic glutamate receptors type 1 peri-synaptic localization at parallel fibre–Purkinje cell synapses

Taking advantage of the dominant-negative property of Homer1a (Tu et al., 1998), we investigated whether this short isoform could alter the distribution of mGlu1 receptors at PF–PC synapses by disrupting their binding with long Homers, hence allowing for higher mobility. Adult mice were infused through the tail vein with 8 mg/kg of either a cell-permeable TAT-conjugated form of Homer1a (TAT-Homer1a; n = 6) or a mutant version unable to bind to mGlu1 receptors (TAT-Homer1aW24Y; n = 6) (Moutin et al., 2012). The two constructs contained a Myc tag, which was used to detect their penetration in PCs by immunofluorescence (Fig. 6A,B).

To further confirm an interaction between mGlu1x receptors and Homer1a, a co-immunoprecipitation experiment using mGlu1x antibodies was carried out on cerebellar detergent lysates obtained from TAT-Homer1a-injected or TAT-Homer1aW24Y-injected mice. Co-immunoprecipitations with mGlu1x antibodies allowed the detection in TAT-Homer1a cerebellar eluates, but not in TAT-Homer1aW24Y, of a band revealed by both anti-Homer1 and c-Myc antibodies and consistent with the TAT-Homer1a protein (Fig. 6C).

These results showed the efficacy of the TAT-Homer1a injection glutamatergic synapses (Fig. 5B,D). The absence of Homer3 labelling at GABAergic synapses (Fig. 5C,E) is thus in line with the hypothesis that long Homer proteins are involved in the peri-synaptic localization of mGlu1 receptors in glutamatergic synapses.

Fig. 6. Detection of TAT-Homer1a in the cerebellar cortex and interaction with mGlu1x receptors. (A) Intense anti-myc immunofluorescence revealing TAT-Homer1a can be observed in PCs, whereas no specific labelling was detected in the control saline-injected mice. (B) Fluorescence intensity was measured across the cerebellar cortex after intravenous injection of TAT-Homer1a or saline to assess the efficiency of cerebellar penetration of the constructs. (C) Co-immunoprecipitation of TAT-Homer1a with mGlu1x receptors from cerebellar detergent lysates of mice injected with TAT-Homer1a or TAT-Homer1aW24Y. The immunoprecipitated samples were subjected to immunoblot analysis in order to detect Myc-tagged TAT-Homer1a. The presence of an additional band (black arrow) above Homer1 (grey arrows, estimated molecular weight 43 kDa) in the TAT-Homer1a lane, but not in the TAT-Homer1aW24Y lane, should be noted. This band was also detected when the same polyvinylidene difluoride membrane was immunolabelled with anti-c-Myc antibodies. The double arrow indicates the band corresponding to the mGlu1x receptor. Arrowheads indicate light-chain IgGs used for immunoprecipitation. Scale bar: 20 μm in A. Data shown in B represent mean ± SD. A.U., arbitrary units.

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because of the known indirect interaction between glutamate δ2 and mGlu1 receptors (Kato et al., 2012; Ohtani et al., 2014), in another set of experiments we analysed, on replicas obtained from the same animals, the glutamate δ2 receptor density in PF–PC synapses. No difference in the synaptic density of glutamate 2 receptors was observed between the two groups of injected mice (n of synapses: TAT-Homer1aW24Y, 163; TAT-Homer1a, 156; Mann–Whitney test, *P < 0.05, ***P < 0.001). (E) The synaptic density of gold particles for Gluδ2 receptors remained unchanged by TAT-Homer1a injection (n of synapses analysed: TAT-Homer1aW24Y, n = 163; TAT-Homer1a, n = 156; Mann–Whitney test, P = 0.167). Data in C–E represent mean ± SEM. Scale bar in A: 200 nm.

Discussion

Here, we provide the first unequivocal demonstration of the intrasynaptic localization of mGlu1 receptors in GABAergic synapses using the FRIL technique, and confirm their peri-junctional enrichment in glutamatergic synapses. Our findings highlight a fundamental difference in the subsynaptic localization of these receptors in neurochemically distinct types of synapse. Moreover, we show that the most abundant long Homer form in the cerebellar cortex, namely Homer3, is exclusively localized in asymmetric glutamatergic synapses consistent with its potential role in accumulating mGlu1 receptors peri-synaptically (Shiraishi-Yamaguchi & Furuichi, 2007). However, the dominant-negative TAT-Homer1a failed to significantly alter the distribution pattern of mGlu1 receptors with respect to the PSD. These findings suggest that the mGlu1–long Homer protein interaction is neither necessary nor sufficient for the peri-junctional accumulation of the receptor.

Homer proteins are known to interact with a proline-rich sequence present in the C-terminus of the mGlu1α receptor variant (Tu et al., 1998), but absent in mGlu1β and mGlu1γ isoforms (Ferraguti et al., 2008). All three splice variants are highly expressed in PCs (Berthele et al., 1998; Ferraguti et al., 2008) and the mGlu1β receptor shows a preferential peri-synaptic distribution similar to mGlu1α (Mateos et al., 2000). So far no localization data are available for the mGlu1β variant, because no immunological tools have yet been developed. As Homer1a can disrupt only the interaction between mGlu1α and long Homer proteins, only a fraction of mGlu1 receptors might have been affected by the injection of TAT-Homer1a. This could have precluded the detection of a significant shift in mGlu1 receptor distribution near PF–PC synapses, in particular if mGlu1 splice variants do not heterodimerize (Remelli et al., 2008), and given that we used a pan-mGlu1 antibody detecting all mGlu1 splice variants. However, we consider this possibility unlikely for a number of reasons. Firstly, the cumulative distribution of the distance of gold particles for mGlu1 receptors from the PSD edge between TAT-Homer1a-injected and TAT-Homer1aW24Y-injected mice was very similar. Secondly, in recent co-immunoprecipitation experiments performed with antibodies against mGlu1α followed by liquid chromatography mass spectrometry, we found unique peptides for both mGlu1α and mGlu1β receptor isoforms in the eluate, suggesting dimerization in vivo (Mansouri and Ferraguti, personal communication; see also Ohtani et al., 2014). Thirdly, TAT-Homer1a also did not influence the synaptic density of δ2 receptors, which are known to be linked, although indirectly, to the C-terminal tail of mGlu1α receptors (Kato et al., 2012; Ohtani et al., 2014), probably through Shank (Uemura et al., 2004).

Although we provide evidence that TAT-Homer1a penetrates in PCs, interacts with mGlu1α, and alters the AMPA receptor density...
at the plasma membrane, our data do not rule out the possibility that TAT-Homer1 was unable to efficiently disrupt the interaction between endogenous Homer proteins and mGlu1 receptors, hence warranting further investigations. Definite proof for a role of Homer proteins in mGlu1 receptor subcellular targeting could be obtained, in our view, only by using genetically modified animals lacking all Homer genes, which, however, have not been developed so far.

Using the highly sensitive FRIL technique, we demonstrate a qualitatively and quantitatively matching mGlu1 subcellular distribution in rat and mouse PCs. Moreover, we show that mGlu1 receptors can be detected in both leaflets of the plasma membrane, suggesting no particular association with lipid subtypes. The planar view of large plasma membrane segments of PC spines, dendrites and somata offered by the FRIL technique allowed us to reveal the widespread extrasynaptic distribution of mGlu1 receptors. With respect to PF–PC synapses, it is noteworthy that we found a very similar frequency distribution of mGlu1 labelling tangential to the PSD as in previous reports, which used more conventional but less sensitive ultrastructural techniques (Baude et al., 1993; Nusser et al., 1994; Lujan et al., 1997; Mateos et al., 2000), when we adopted the same bin width (60 nm).

Previous studies suggested that mGlu1 receptors could reside in the main body of type II symmetric synapses on pallidal and nigral neurons (Hanson & Smith, 1999; Hubert et al., 2001). Our findings extend this work and provide conclusive evidence for the intrasy- naptic localization of mGlu1 receptors in GABAergic synapses onto PCs as well as on pallidal neurons. The direct presence of mGlu1 receptors in GABAergic synapses raises important questions concerning their functional role as well as about the source of glutamate needed for their activation.

Several forms of plasticity of inhibitory transmission have been reported to occur in cerebellar PCs, which include RP and DSI (Kano et al., 1992; Galante & Diana, 2004). Both DSI and RP were shown to require activation of mGlu1 receptors (Galante & Diana, 2004; Sugiyama et al., 2008). Although depolarization-induced elevations of intracellular Ca2+ can lead to both depolarization-induced suppression of excitation and DSI, the induction of DSI by mGlu1 receptors was independent of post-synaptic Ca2+ increases and suppression of excitation and DSI, the induction of DSI by mGlu1 receptors by glutamate released from PFs (Galante & Diana, 2004). The current hypothesis posits that 1 cannabinoid receptors, which in turn inhibit neurotransmitter invasions of intracellular Ca2+ can lead to both depolarization-induced extrasynaptic suppression of excitation and DSI, the indirect mechanism being most widely described (Kano et al., 1994; Sugiyama et al., 1992; Lujan et al., 1997; Mateos et al., 2000), when we adopted the same bin width (60 nm).

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Abbreviations

DSI, depolarization-induced suppression of inhibition; FRIL, freeze-fracture replica immunogold labelling; IMP, intramembrane particle; KO, knockout; mGlu1, metabotropic glutamate receptor type 1; PB, phosphate buffer; PBS, phosphate-buffered saline; PC, Purkinje cell; PF, parallel fibre; P-face, protoplasmic face; PSD, post-synaptic density; RP, rebound potentiation; RT, room temperature; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing 0.1% Triton X-100.

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