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POSTNATAL INDUCTION AND LOCALIZATION OF R7BP, A MEMBRANE-ANCHORING PROTEIN FOR REGULATOR OF G PROTEIN SIGNALING 7 FAMILY-Gβ5 COMPLEXES IN BRAIN

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Abstract—Members of the regulator of G protein signaling 7 (RGS7) (R7) family and Gβ5 form obligate heterodimers that are expressed predominantly in the nervous system. R7-Gβ5 heterodimers are GTPase-activating proteins (GAPs) specific for Gi/o-class Gα subunits, which mediate phototransduction in retina and the action of many modulatory G protein-coupled receptors (GPCRs) in brain. Here we have focused on the R7-family binding protein (R7BP), a recently identified palmitoylated protein that can bind R7-Gβ5 complexes and is hypothesized to control the intracellular localization and function of the resultant heterotrimeric complexes. We show that: 1) R7-Gβ5 complexes are obligate binding partners for R7BP in brain because they co-immunoprecipitate and exhibit similar expression patterns. Furthermore, R7BP and R7 protein accumulation *in vivo* requires Gβ5. 2) Expression of R7BP in Neuro2A cells at levels approximating those in brain recruits endogenous RGS7-Gβ5 complexes to the plasma membrane. 3) R7BP immunoreactivity in brain concentrates in neuronal soma, dendrites, spines or unmyelinated axons, and is absent or low in glia, myelinated axons, or axon terminals. 4) RGS7-Gβ5-R7BP complexes in brain extracts associate inefficiently with detergent-resistant lipid raft fractions with or without G protein activation. 5) R7BP and Gβ5 protein levels are upregulated strikingly during the first 2–3 weeks of postnatal brain development. Accordingly, we suggest that R7-Gβ5-R7BP complexes in the mouse or rat could regulate signaling by modulatory Gi/o-coupled GPCRs in the developing and adult nervous systems.

Key words: postnatal brain development, signal transduction, palmitoylation, lipid raft, G protein-coupled receptors, immunohistochemistry.

G protein–coupled receptors (GPCRs) regulate neuronal structure and function by controlling ion channel activity, second messenger production and protein kinase activation (Gainetdinov et al., 2004; Schubert et al., 2006). Modulatory GPCR signaling pathways are tightly controlled by mechanisms such as GPCR phosphorylation and arrestin binding (Lefkowitz and Shenoy, 2005; Dhami and Ferguson, 2006).

GPCR signaling regulation also involves the action of RGS proteins (*r*egulators of *G* protein *s*ignaling), which accelerate GTP hydrolysis by Gα subunits (i.e. RGS proteins are GTPase-activating proteins or GAPs (Berman et al., 1996; Hunt et al., 1996; Watson et al., 1996)). The regulator of G protein signaling 7 (RGS7) (R7) family (RGS6, RGS7, RGS9-1, RGS9-2 and RGS11) is thought to be particularly important for regulating GPCR function in the nervous system. R7 family members form obligate heterodimers with Gβ5 (Cabrera et al., 1998; Snow et al., 1998; Makino et al., 1999; He et al., 2000; Kovoor et al., 2000; Witherow et al., 2000; Zhang and Simonds, 2000; Chen et al., 2003), and are expressed significantly only in retina and the nervous system (Witherow et al., 2000; Jones et al., 2004). R7-Gβ5 heterodimers have GAP activity specific for Gi/o-class α subunits (Hooks et al., 2003), which mediate phototransduction and modulatory GPCR signaling. RGS9 is the best understood R7 family member. Genetic inactivation of RGS9 slows the termination kinetics of phototransduction in mice (Chen et al., 2000; Krispel et al., 2003), and causes light adaptation and contrast detection defects in humans (Nishiguchi et al., 2004). RGS9 knockout mice also exhibit augmented response to opioids and cocaine (Rahman et al., 2003; Zachariou et al., 2003).

The R7-family binding protein, R7-family binding protein (R7BP), was identified recently as a protein that can interact with R7-Gβ5 heterodimers to form heterotrimeric complexes (Drenan et al., 2005; Martemyanov et al., 2005). When palmitoylated, overexpressed R7BP directs R7-Gβ5-R7BP heterotrimers to the plasma membrane, whereas when depalmitoylated it shuttles them into the nucleus of transiently transfected cells (Drenan et al., 2005; Song et al., 2006). R7BP palmitoylation and plasma membrane targeting augment the ability of RGS7-Gβ5-R7BP complexes to accelerate the kinetics of Gi/o-mediated signaling (Drenan et al., 2005, 2006).

Despite such information, little is known about the function of R7BP in the nervous system. For example, it is unknown whether R7-Gβ5 complexes are obligate binding partners for R7BP *in vivo*, whether R7BP is expressed in

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Abbreviations: Ara-c, cytosine arabinoside; DIV, days *in vitro*; DRM, detergent-resistant membrane; GAP, GTPase-activating protein; GFAP, glial fibrillary acidic protein; GPCR, G protein–coupled receptor; NeuN, neuronal nuclear antigen; N2a, Neuro2a; RGS, regulator of G protein signaling; RGS7, R7, regulator of G protein signaling 7; R7BP, R7-family binding protein.

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neurons and/or glia, or whether expression of R7BP at normal levels in neuronal cells is sufficient to recruit endogenous R7-Gβ5 complexes to the plasma membrane. Likewise, little is known about the regional, subcellular or developmental expression of R7BP in brain, which would suggest when or where R7-Gβ5-R7BP complexes could regulate signaling by modulatory GPCRs. Here we have addressed these questions in order to better understand the function of R7-Gβ5-R7BP complexes in brain.

EXPERIMENTAL PROCEDURES

Vertebrate animals

All procedures involving vertebrate animals were performed in strict accord with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Study Committees of Washington University School of Medicine, Emory University School of Medicine and the University of Miami School of Medicine. The number of animals used was the minimum required for data replication. Standard procedures were used to prevent animal suffering. Mice were housed in animal facilities with free access to water and food, and with a 12-h light/dark cycle. A breeding pair of Gβ5−/+ mice in a mixed genetic background was generously provided to one of us (V.Z.S.) by Dr. Jason Chen (Virginia Commonwealth University, Richmond, VA, USA). These mice were backcrossed with C57BL/6J mice until the F3 generation of Gβ5−/+ mice was obtained. Gβ5−/+ mice were interbred to obtain Gβ5−/−, Gβ5−/+ and wild type mice. Neonatal rat pups were obtained by purchasing timed pregnant Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA). Except for studies involving Gβ5−/− mice, all wild type mice used were of the pure C57BL/6 (Taconic Laboratories, Hudson, NY, USA) strain.

Generation of affinity-purified chicken and rabbit anti-R7BP antibodies

MBP and GST fusion proteins bearing full-length mouse R7BP at their C-termini were expressed and purified from derivatives of pMAL-C2H10T and pGEX-2T in *E. coli* strain BL2DE3-pLysS. MBP-R7BP (>90% purity) was used to immunize chickens (Aves Laboratories, Tigard, OR, USA) and rabbits (Zymed Laboratories, Carlsbad, CA, USA). The resultant antibodies were affinity purified by the commercial antibody providers on columns coupled to GST-R7BP. The concentrations of affinity-purified R7BP antibody stocks were 0.7 mg/ml (chicken antibody) and 0.21 mg/ml (rabbit antibody); these stocks were diluted as described below for use in various experiments.

Confocal fluorescence immunohistochemistry

Mice were anesthetized deeply (87 mg/kg ketamine HCl, 13.4 mg/kg xylazine HCl i.p.) and perfused intracardially with PBS followed by 4% paraformaldehyde in PBS. Brain was removed and kept overnight in 4% paraformaldehyde at 4 °C. For immunohistochemistry experiments using free floating sections (40 μm-thick), brains were cryoprotected using 30% sucrose in PBS at 4 °C and sections were cut using a sliding microtome. Samples then were processed for immunohistochemistry according to instructions provide by the manufacturer of the Vectastain kit (Vector Laboratories, Burlingame, CA, USA). Briefly, tissue sections were rinsed in PBS and endogenous peroxidase was inactivated by treating with 0.04%Triton X-100 in PBS containing 0.5% H_2O_2 for 1 h. To block non-specific antibody binding, sections were incubated in blocking buffer (5% non-fat milk, 1% normal goat serum in PBS) for 1 h. Sections then were incubated at 4 °C overnight with affinity purified anti-R7BP antibodies (1:2000, chicken antibody; 1:1000 rabbit antibody, each in blocking buffer). After several rinses with PBS, sections were incubated 1 h at room temperature with biotinylated secondary anti-chicken or anti-rabbit antibody (1:1000; Vector Laboratories), rinsed in PBS and incubated for 1 h with avidin–biotin–HRP complex (ABC Vectastain kit, Vector Laboratories). After several rinses in TBS, sections were incubated for 10 min in 0.05% diaminobenzidine (Sigma, St. Louis, MO, USA) and 0.015% hydrogen peroxide, rinsed in PBS, mounted, dried, dehydrated and embedded in Cytoseal 60 medium under coverslips. Blocked anti-R7BP antibodies were prepared by incubating affinity-purified chicken or rabbit antibodies (1 μl antibody stock diluted in 1–200 μl of blocking buffer) with MBP-R7BP fusion protein (16.5 μg) for 2 h at 37 °C. Regional distribution of R7BP was analyzed with the aid of the rodent brain atlas (Paxinos and Watson, 1982). For experiments using thin (10 μm) sections of brain, we embedded tissue in paraffin before sectioning. For immunofluorescence labeling, paraffin-embedded sections were rehydrated and permeabilized for 25 min with trypsin (1 mg/ml trypsin tablet for immunohistochemistry; Sigma) in PBS. Sections were washed with PBS and incubated at 4 °C overnight with chicken or rabbit anti-R7BP antibody (1:100 in blocking buffer) and co-stained overnight with monoclonal mouse anti-neuronal nuclear antigen (NeuN, Chemicon International Inc., Temecula, CA, USA; 1:100) or with polyclonal rabbit anti-glial fibrillary acidic protein (GFAP; Sigma) antibody (1:150). After several rinses in PBS, sections were incubated 1 h at room temperature with biotinylated secondary anti-chicken, anti-rabbit and/or anti-mouse antibodies (Vector Laboratories) diluted 1:1000 in PBS, rinsed in PBS and incubated for 1 h in avidin–biotin–HRP complex (ABC Vectastain kit, Vector Laboratories). Sections were visualized by using the TSA Plus amplification system (NEN Life Science Products, Boston, MA, USA), which converts an HRP substrate to a fluorescent product. Sections were mounted on slides for confocal microscopy (model LSM-510; Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). In each experiment we used sections from brain obtained from at least three animals.

Electron microscopic immunocytochemistry

Five adult rats were perfusion-fixed with a mixture of 4% paraformaldehyde/0.1% glutaraldehyde and used for electron microscopic localization of R7BP protein in the striatum and the thalamus, two brain regions significantly enriched in R7BP immunoreactivity. Following perfusions, brains were removed from the skull, post-fixed in 4% paraformaldehyde for 2–24 h, cut into 60-μm-thick sections using a vibrating microtome and stored in PBS at 4 °C until processed for immunocytochemistry. Prior to immunocytochemical processing, all sections were put into a 1% sodium borohydride solution for 20 min and then washed with PBS.

Following sodium borohydride treatment, sections were placed in a cryoprotectant solution (0.05 M Na-phosphate, pH 7.4, 25% sucrose, and 10% glycerol) for 20 min, frozen at −80 °C for 20 min, returned to a decreasing gradient of cryoprotectant solutions, and rinsed in PBS. Sections were then incubated in primary and secondary antibody solutions, identical to those used for light microscopy, with two exceptions: 1) the omission of Triton X-100 and 2) incubation in primary antibody for 48 h at 4 °C.

After the DAB reaction, the tissue was rinsed in PB (0.1 M, pH 7.4) and treated with 1% OsO_4 for 20 min. It was then returned to PB and dehydrated with increasing concentrations of ethanol. When exposed to 70% ETOH, 1% uranyl acetate was added to the solution for 35 min to increase the contrast of the tissue in the electron microscope. Following dehydration, sections were treated with propylene oxide and embedded in epoxy resin for