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Regional Differences in the Coupling of 5-Hydroxytryptamine-1A Receptors to G Proteins in the Rat Brain

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ABSTRACT

Numerous data showed that 5-hydroxytryptamine-1A (5-HT_{1A}) receptors couple to $G\alpha_o/\alpha_i$ proteins for signal transduction. However, the α subunit isoforms really involved in 5-HT_{1A} receptor coupling in brain remain to be identified. Moreover, regional differences in the functional characteristics of brain 5-HT_{1A} receptors have been evidenced repeatedly. Because such differences could be due to variations in G proteins interacting with the same receptor, relevant approaches were used for identifying α subunits physically coupled to 5-HT_{1A} receptors in different regions of the rat brain. Using immunoaffinity chromatography coupled to Western blot detection, 5-HT_{1A} receptors were found to interact equally with $G\alpha_o$ and $G\alpha_{i3}$ in the cerebral cortex, mainly with $G\alpha_o$ and weakly with $G\alpha_{i3}$ in the hippocampus and exclusively with $G\alpha_{i3}$ in the anterior raphe area. In the hypothalamus, 5-HT_{1A} receptors seemed to be coupled to the latter two G proteins plus $G\alpha_{i1}$ and $G\alpha_z$. Complementary experiments based on an antibody capture technique coupled to both classic radioactivity and scintillation proximity assay detections showed that hippocampal 5-HT_{1A} receptor stimulation induced 5′-*O*-(3-[^{35}S]thio)triphosphate binding to immunoprecipitates with $G\alpha_{i3}$ and $G\alpha_o$ antisera. In the anterior raphe, such 5-HT_{1A} receptor-mediated effect was obtained with $G\alpha_{i3}$ antiserum only. These results demonstrated the existence of regional differences in the coupling of 5-HT_{1A} receptors to G proteins in the rat brain. In the anterior raphe, 5-HT_{1A} receptors seem to interact specifically with $G\alpha_{i3}$, whereas in the hippocampus, they are mainly coupled to $G\alpha_o$ proteins. Such a disparity in G-protein coupling might explain regional differences in adaptive regulations of brain 5-HT_{1A} receptors.

Because of its involvement in various psychiatric pathologies, such as affective disorders, the 5-HT_{1A} type of serotonin (5-hydroxytryptamine, 5-HT) receptors is a subject of great interest. 5-HT_{1A} receptor stimulation has been shown to activate different signaling pathways sensitive to pertussis toxin through $G\alpha_i/G\alpha_o$ proteins (Raymond et al., 1993). Inhibition of adenylyl cyclase (AC) activity mediated by 5-HT_{1A} receptors has been evidenced in most brain regions as a result of $G\alpha_i$ protein activation (De Vivo and Maayani, 1986; Hamon, 1997). In addition, in the hippocampus, 5-HT_{1A} receptors have been shown to mediate G-protein-gated inwardly rectifying K^+ current (GIRK) through a direct interaction of the ionic channel with the $\beta\gamma$ subunits of the $G\alpha_o$ isoform (Andrade and Nicoll, 1987; Oleskevich, 1995).

The development of heterologous recombinant systems expressing the 5-HT_{1A} receptor, such as COS-7, HeLa, Chinese hamster ovary, Sf9, GH4C1, LLC-PK1, and NIH-3T3 transfected cells, has provided relevant models to study the receptor coupling with different G-protein subtypes and to identify

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ABBREVIATIONS: 5-HT_{1A}, 5-hydroxytryptamine-1A; 5-CT, 5-carboxamidotryptamine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; AC, adenylyl cyclase; 5-HTT, 5-hydroxytryptamine transporter; [^{35}S]GTPγS, 5′-*O*-(3-[^{35}S]thio)triphosphate; DRN, dorsal raphe nucleus; PBS, phosphate-buffered saline; RGS, regulator of G-protein signaling; SSRI, selective serotonin reuptake inhibitor; GIRK, G-protein-gated inwardly rectifying K^+; SPA, scintillation proximity assay; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin; WAY 100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexane carboxamide.

second messengers. These studies led to the conclusion that 5-HT_{1A} receptors preferentially interact with $G\alpha_{i3}$ subunits, followed, in decreasing affinity order, by $G\alpha_{i2}$ and less strongly with $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_z$ proteins (Fargin et al., 1991; Bertin et al., 1992; Liu et al., 1994; Garnovskaya et al., 1997; Newman-Tancredi et al., 2002). In contrast, 5-HT_{1A} receptor coupling to $G\alpha_q$ and $G\alpha_s$ seemed to be weak or absent in such heterologous recombinant systems (Raymond et al., 1993).

The complexity of 5-HT_{1A} receptor coupling, evidenced in recombinant systems, matches the agonist-directed trafficking of receptor signaling theory (Kenakin, 1995). This concept suggests that, depending on the nature of the agonist used, receptors will selectively activate one specific G-protein subtype and downstream transduction pathway. Moreover, this theory also stresses the influence of the "receptor/G-protein ratio" on both the nature of the G protein involved in receptor signaling and the agonist efficacy (Kenakin, 1995; Newman-Tancredi et al., 1997).

In this context, it is well-established that 5-HT_{1A} receptor ligands may act as full agonists in the dorsal raphe nucleus (DRN) but only as partial agonists in the hippocampus (Sprouse and Aghajanian, 1988). Another example of 5-HT_{1A} receptor functional heterogeneity that might also be relevant to this theory concerns the regional differences in 5-HT_{1A} receptor adaptive changes caused by long-term modifications in central 5-HT neurotransmission. Thus, long-term treatment with selective serotonin reuptake inhibitors (SSRIs) and 5-HT transporter (5-HTT) gene disruption are well-known to induce functional desensitization of 5-HT_{1A} autoreceptors in the DRN but no changes in postsynaptic 5-HT_{1A} sites in the hippocampus (Chaput et al., 1986; Le Poul et al., 2000; Mannoury la Cour et al., 2001). In the DRN, this adaptive regulation is associated with a decrease in 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding, suggesting an alteration of the receptor/G-protein coupling in both SSRI-treated rodents (Hensler, 2002) and 5-HTT knockout mice (Fabre et al., 2000). In the hypothalamus, the 5-HT_{1A} receptor desensitization that occurs in these two models was reported to be associated with down-regulation of $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_z$ proteins (Li et al., 1997; Raap et al., 1999). Taken together, these data support the idea that such regional differences in the functional and adaptive properties of brain 5-HT_{1A} receptors are probably underlain by variations in receptor coupling to G proteins from one area to another.

To assess this hypothesis, we applied combined immunoaffinity chromatography and immunoblotting approaches. A specific anti-rat 5-HT_{1A} receptor antiserum was used (El Mestikawy et al., 1990; Riad et al., 1991) to identify the $G\alpha$ subunits physically coupled to 5-HT_{1A} receptors in membrane preparations from different brain regions. Furthermore, $G\alpha$ subunits concerned by 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding were determined using both antibody capture assay with protein A-Sepharose beads and scintillation proximity assay (SPA) (Cussac et al., 2002).

Materials and Methods

Experiments were performed using adult male Sprague-Dawley rats (250–400 g body weight; Centre d'Elevage René Janvier, Le Genest-Saint-Isle, France). Animals were maintained under standard laboratory conditions (22 ± 1°C, 60% relative humidity, 12-h light/dark cycle, food and water ad libitum) for 5 to 10 days before the beginning of experiments. All the procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council directive 87-848, 1987 October 19, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions number 75-116 to M.H. and 75-977 to L.L).

Preparation of Membranes. Rats were killed by decapitation. Their brains were quickly removed, and the cerebral cortex, striatum, hippocampus, cerebellum, hypothalamus, and anterior raphe area were dissected in cold (0–4°C) and stored at −80°C before use. Frozen tissues were homogenized in 10 volumes (v/w) of ice-cold 0.05 M Tris-HCl containing 0.01 mM phenylmethylsulfonyl fluoride and 0.01 mM aprotinin, pH 7.4, with a Polytron tissue disrupter (PT OD; Kinematica, Basel, Switzerland). Homogenates were centrifuged at 40,000*g* for 20 min at 4°C. The pellets were washed twice by resuspension in 40 volumes of the same ice-cold buffer, followed by centrifugation and homogenization in the same volume of buffer. The resulting membrane suspension was incubated for 10 min at 37°C to allow the dissociation of membrane-bound endogenous 5-HT and then centrifuged and washed three more times as described above. The final pellet was gently homogenized in an appropriate volume of 0.05 M Tris-HCl, pH 7.4, to obtain the membrane suspension (~20 mg of membrane proteins per milliliter) to be stored at −80°C until use.

Solubilization Procedure. Thawed membrane suspension was mixed with 0.1 volume (v/v) of 0.1 M [0.6% (w/v)] CHAPS in 0.05 M Tris-HCl, pH 7.4, then briefly sonicated (20 W/5 s) and left for 60 min at 4°C (El Mestikawy et al., 1988). The mixture was then centrifuged at 100,000*g* for 30 min at 4°C. The clear supernatant was collected and filtered through a 0.22-μm Millex GV filter (Millipore Corporation, Billerica, MA) before its use as the source of solubilized 5-HT_{1A} binding sites in subsequent assays. The protein concentration in the final soluble extract was ~6 mg/ml.

Immunoaffinity Chromatography, Elution, and Concentration Procedures. The anti-rat 5-HT_{1A} receptor polyclonal antibody was purified (El Mestikawy et al., 1990; Riad et al., 1991) and coupled to an Affigel-10 column (2 cm high, 1.5 cm in diameter) as recommended by the manufacturer (Bio-Rad, Hercules, CA). The filtered supernatant (~4 ml) from CHAPS-treated membranes was poured into the affinity column equilibrated with 0.05 M Tris-HCl, pH 7.4. After an overnight incubation at 4°C, the supernatant was removed, and the column was washed four times with 40 ml of 0.05 M Tris-HCl, pH 7.4, containing 0.1 M CHAPS and then with the same volume of the same buffer containing 0.01 M CHAPS.

Elution was performed in two steps. First, the $G\alpha$ protein subunits specifically coupled to 5-HT_{1A} receptors were eluted within 15 min, at room temperature, with 5 ml of 0.05 M Tris-HCl, pH 7.4, supplemented with 1 mM 5-HT and 1 mM GTP. The column was then washed with 4 × 20 ml of 0.05 M Tris-HCl buffer, and the second elution was made at 4°C with 5 ml of 0.01 M glycine-HCl, pH 2, containing 0.01 M CHAPS to collect 5-HT_{1A} receptors adsorbed onto the column. Eluate was immediately neutralized with 1 M Tris-HCl, pH 7.4, and dialyzed against 2 liters of 0.05 M Tris-HCl, pH 7.4, containing 0.1% SDS, using a MicroProDiCon apparatus (model MPDC-310; Bio Molecular Dynamics, Beaverton, OR). After 3 days at 4°C, the neutralized eluate was concentrated to a final volume of 300 μl. The same dialysis-concentration procedure was applied to the first eluted fraction (5 ml) containing $G\alpha$ proteins.

Immunoblot Analysis of Eluted $G\alpha$ Proteins and 5-HT_{1A} Receptors. The solubilized proteins in concentrated dialysates were analyzed by SDS-polyacrylamide gel electrophoresis using 0.5-mm thick 10% acrylamide/bisacrylamide [29:1 (w/w)] gels with 0.1% SDS and 0.375 M Tris, pH 8.8. After migration, the proteins were electrophoretically transferred for 45 min to a nitrocellulose membrane (Hybond ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) that was then incubated, at room temperature, in PBS/0.1%

Tween (v/v) containing 5% nonfat dry milk for 1 h. The membrane was subsequently incubated overnight with rabbit polyclonal antibodies directed against either Gα_o, Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_z, or Gα_s (1:200 dilution) at 4°C. Analysis of the specificity of these anti-Gα antibodies using recombinant Gα subunits showed that no cross-reactivity occurred at the dilution used in our experiments except for anti-Gα_{i1} and anti-Gα_{i3}, which slightly cross-reacted with Gα_{i3} and Gα_{i1} proteins, respectively. The secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase conjugate; Sigma, St. Louis, MO) (1:16,000 dilution) was applied to the membrane for 60 min at room temperature. The blot was washed five times with PBS containing 0.1% Tween and once with PBS alone. After a 5-min incubation in ECL Plus chemiluminescence substrate solution (GE Healthcare), the membrane was exposed to autoradiography Hyperfilm MP (GE Healthcare) for ~15 s or analyzed using filmless autoradiographic analysis (FLA2000; Fuji, Tokyo, Japan).

Protein Determination. Proteins were estimated using the Folin phenol procedure (Lowry et al., 1951) with bovine serum albumin as the standard.

[^{35}S]GTPγS Binding and Gα Subunit-Specific Immunoprecipitation. The binding of [^{35}S]GTPγS to specific Gα proteins upon activation of 5-HT$_{1A}$ receptors was measured using a method adapted from Selkirk et al. (2001). A first series of experiments allowed the determination of the optimal assay conditions leading to the highest ratio of 5-HT$_{1A}$-enhanced over basal [^{35}S]GTPγS binding when starting with hippocampal membranes. On this basis, brain membranes (0.2–1.0 mg/ml) were incubated for 30 min in assay buffer (67 mM Tris-HCl, 4 mM $MgCl_2$, 160 mM NaCl, and 0.267 mM EGTA, pH 7.4) containing GDP (1.2 mM), [^{35}S]GTPγS (0.4 nM), with or without 5-carboxamidotryptamine (5-CT, 10 μM) and WAY 100635 (10 μM, for the determination of nonspecific [^{35}S]GTPγS binding; Fabre et al., 2000) (final volume, 800 μl) in a shaking water bath at 37°C. Incubation was ended by the addition of 500 μl of ice-cold assay buffer and transfer to ice. Membranes were separated from the reaction mix by centrifugation at 20,000*g* for 6 min, and the supernatant was discarded. Membrane pellets were then solubilized with 500 μl of a solubilization buffer [100 mM Tris-HCl, 1 mM EDTA, 20 mM NaCl, and 0.62% (v/v) Nonidet P-40, pH 7.4] for 1 h at 4°C. Samples were centrifuged (20,000*g*, 4°C), and 400 μl of the supernatant was incubated with anti-Gα protein antiserum (1/100) during 90 min at 4°C. Protein A-Sepharose beads [70 μl, 50% (w/v)] were added, and samples were rotated for a further 90 min at 4°C. After centrifugation (20,000*g*, 4°C), the supernatant was removed by aspiration, and the beads were washed three times with 500 μl of solubilization buffer and then resuspended in the same solubilization buffer (500 μl) and filtered through Whatman GF/B filters. After three washes with ice-cold 67 mM Tris-HCl, pH 7.4, each filter was immersed in 5 ml of scintillation fluid, and the entrapped radioactivity was counted. Data are expressed as mean ± S.E.M. of at least three independent experiments.

Scintillation Proximity Assays. SPAs were used to further determine G-protein subtypes specifically activated by 5-HT$_{1A}$ receptor stimulation. The procedure described by Cussac et al. (2002) was adapted so as to be used after [^{35}S]GTPγS binding and solubilization steps (see above). After solubilization, samples (200 μl) were transferred into a 96-well Opti plate (PerkinElmer Life and Analytical Sciences, Boston, MA) and incubated with 2 μl of specific anti-Gα polyclonal antibody (1/100) during 1 h at room temperature. SPA beads coated with secondary anti-rabbit antibodies (GE Healthcare) were then added in a volume of 50 μl/well. After overnight incubation under gentle agitation, the plates were centrifuged (10 min at 1300*g*), and radioactivity entrapped on beads was measured using a TopCount microplate scintillation counter (PerkinElmer Life Sciences). Data are expressed as mean ± S.E.M. of four independent experiments.

Chemicals. The following drugs were used: [^{35}S]GTPγS (1000 Ci/mmol), from GE Healthcare; CHAPS, Nonidet P-40, phenylmethylsulfonyl fluoride, aprotinin, 5-HT creatinine sulfate, *R*-(+)-8-OH-DPAT HBr, and (±)-8-OH-DPAT HBr, from Sigma-Aldrich; 5-CT, from RBI/Sigma (Natick, MA); WAY 100635, from Wyeth-Ayerst (Princeton, NJ); GDP dilithium salt and GTP from Roche (Meylan, France); rabbit polyclonal antibodies directed against rat Gα_o, Gα_{i1}, and Gα_{i3}, human Gα_{i2}, Gα_z, and Gα_s, and mouse Gα_q (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis. All experiments (immunoaffinity chromatography, immunoblot analysis of eluted Gα proteins and 5-HT$_{1A}$ receptors, [^{35}S]GTPγS binding, and Gα subunit-specific immunoprecipitation, SPA) have been replicated at least three times (three to eight times) in independent trials.

Data were analyzed using paired Student's *t* test with the help of Prism 4 software (GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p \leq 0.05$.

Results

Immunoaffinity Chromatography Identification of Gα Subunits Specifically Coupled to 5-HT$_{1A}$ Receptors

Regional Distribution of Gα_o, Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_z, and Gα_s Subunits. The presence of Gα-protein subtypes in membranes from the different rat brain regions of interest was investigated using an immunoblotting technique. Rabbit polyclonal antiserums used in these experiments were raised against peptide sequence in highly divergent domains of Gα subunits from rat or human. As shown in Fig. 1, Gα_o, Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_z, and Gα_s proteins were present in all brain structures studied (hippocampus, anterior raphe area, cortex, and hypothalamus), including striatum and cerebellum, in which 5-HT$_{1A}$ receptors are not detected (Riad et al., 1991).

Immunoblotting with anti-Gα_o (Fig. 1A), anti-Gα_s (Fig. 1B), and anti-Gα_{i3} (Fig. 1F) yielded only one band at 45, 47.5, and 40 kDa, respectively. These molecular masses matched those reported in the literature for the three Gα protein subtypes (Schandar et al., 1998). In contrast, a doublet was found with anti-Gα_z (Fig. 1C), anti-Gα_{i1} (Fig. 1D), and anti-Gα_{i2} (Fig. 1E), the second minor band probably corresponding to a nonspecific signal (Allouche et al., 1999).

Evidence for 5-HT$_{1A}$ Receptor Retention on the Immunoaffinity Column. To validate its capacity to bind 5-HT$_{1A}$ receptors, the immunoaffinity column was loaded with hippocampal CHAPS-solubilized extracts, and glycine-HCl/CHAPS, pH 2, eluates were analyzed by immunoblotting with anti-5-HT$_{1A}$ receptor antibodies. As shown in Fig. 2, these antibodies labeled a single diffuse band at ~63 kDa in crude hippocampal extracts (Hip), corresponding to the molecular mass of native *N*-glycosylated 5-HT$_{1A}$ receptor (Emerit et al., 1987; El Mestikawy et al., 1989; Riad et al., 1991). The same heavily labeled band was found in the glycine-HCl fraction eluted from the immunoaffinity column (Fig. 2).

Regional Differences in Gα-Proteins Specifically Coupled to 5-HT$_{1A}$ Receptors. Previous studies have established that neither the solubilization procedure nor antibody binding onto the receptor alters the coupling of 5-HT$_{1A}$ receptor to G protein (El Mestikawy et al., 1988; Emerit et al., 1990). 5-HT$_{1A}$ receptor-G-protein complexes were selectively adsorbed onto the immunoaffinity column, and G proteins were dissociated from these complexes by elution with a mix of 5-HT (1 mM) and GTP (1 mM). Immunoblotting with specific anti-Gα subunit antibodies revealed the presence of Gα_o and Gα_{i3}, but not Gα_{i1}, in eluates from immunoaffinity

column loaded with cortical 5-HT_{1A}-G protein complexes (Fig. 3A). Similar data were obtained with hippocampal 5-HT_{1A} receptor-G protein complexes (Fig. 3B). However, for the hippocampus, $G\alpha_o$ was more intensely labeled than $G\alpha_{i3}$. For the anterior raphe area, only $G\alpha_{i3}$ could be detected in eluates from immunoaffinity column loaded with 5-HT_{1A} receptor-G-protein complexes from this region (Fig. 3C). Concerning hypothalamic 5-HT_{1A} receptor-G protein complexes, in addition to $G\alpha_o$ and $G\alpha_{i3}$ subunits, we also identified $G\alpha_{i1}$ and $G\alpha_z$ in corresponding immunoaffinity column eluates (Fig. 3D).

In contrast, immunoblotting analyses of immunoaffinity column eluates yielded no labeling with anti-$G\alpha_{i2}$, anti-$G\alpha_z$, and anti-$G\alpha_s$ in case of 5-HT_{1A} receptor-G protein complexes solubilized from cortical (Fig. 3A), hippocampal (Fig. 3B), and anterior raphe (Fig. 3C) membranes. No immunolabeling with anti-$G\alpha_s$ and anti-$G\alpha_{i2}$ was also noted with receptor complexes solubilized from the hypothalamus (Fig. 3D).

As expected from the absence of 5-HT_{1A} receptors in the striatum and the cerebellum (Hamon, 1997), no $G\alpha$ proteins could be detected in eluates from immunoaffinity columns loaded with soluble membrane extracts from these regions (Fig. 3, A–D).

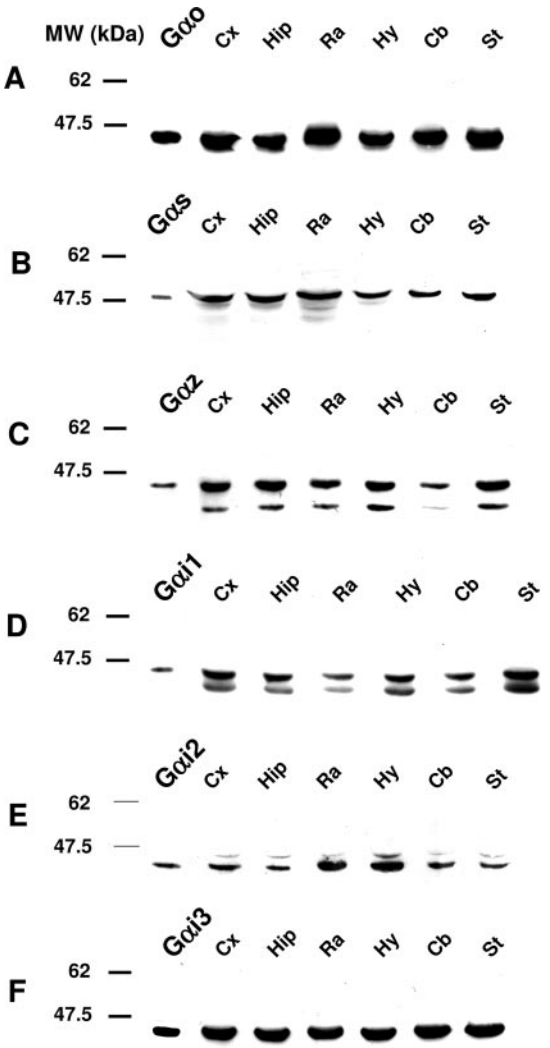

Fig. 1. Immunoblots of $G\alpha$ subunits in membranes from various rat brain regions. Membrane-bound proteins were solubilized by CHAPS, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes as described under *Materials and Methods*. The latter membranes were incubated overnight with 1:200 dilution of antisera against $G\alpha_o$ (A), $G\alpha_s$ (B), $G\alpha_z$ (C), $G\alpha_{i1}$ (D), $G\alpha_{i2}$ (E), and $G\alpha_{i3}$ (F). Molecular mass markers, in kilodaltons, are indicated on the left. The first band (from left to right) corresponds to the recombinant protein and the others to various brain structures: Cx, cerebral cortex; Hip, hippocampus; Ra, anterior raphe area; Hy, hypothalamus; Cb, cerebellum; St, striatum.

Identification of G-Protein Subtypes Activated by 5-HT_{1A} Receptor Stimulation in Various Rat Brain Regions

5-HT_{1A} Receptor Agonist-Induced [^{35}S]GTPγS Binding onto Soluble Extracts from Rat Brain Membranes. Under optimal conditions determined from a preliminary series of experiments (see *Materials and Methods*), [^{35}S]GTPγS binding induced by 10 μM 5-CT reached ~60% over basal with hippocampal membranes (Fig. 4, A and B). The percentage of increase produced by 5-CT was less with membranes from the cerebral cortex and the anterior raphe area (Fig. 4A), in line with the lower density of 5-HT_{1A} receptors in these two regions compared with the hippocampus (Fabre et al., 2000).

In addition to 5-CT, the full agonist *R*-(+)-8-OH-DPAT also induced a marked increase in [^{35}S]GTPγS binding onto soluble extracts from hippocampal membranes (+48.6 ± 1.9% over basal, mean ± S.E.M., n = 3). In contrast, the partial 5-HT_{1A} agonist, (±)-8-OH-DPAT (10 μM), only produced a minor effect (+12%), and the 5-HT_{1A} receptor antagonist, WAY 100635 (5 nM–10 μM), was completely ineffective (Fig. 4, B and C). However, the latter compound inhibited, in a concentration-dependent manner (IC_{50} = 50 ± 17 nM), [^{35}S]GTPγS binding stimulation induced by 10 μM 5-CT (Fig. 4C), further confirming that 5-HT_{1A} receptor activation entirely accounted for 5-CT-evoked increase in [^{35}S]GTPγS binding under such assay conditions.

Immunoprecipitation of Gα Proteins Labeled with [^{35}S]GTPγS in Soluble Extracts from 5-CT-Stimulated Hippocampal Membranes. In a first series of experiments, protein A-Sepharose beads were used to bind immunoprecipitates with various anti-$G\alpha$ antibodies of [^{35}S]GTPγS-labeled

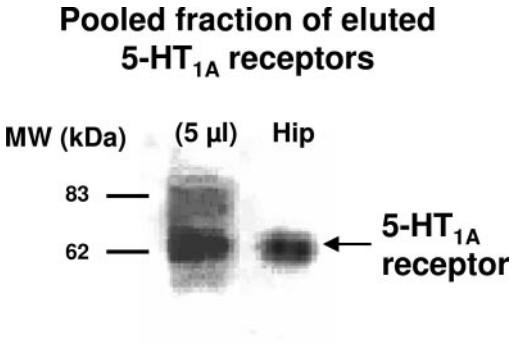

Fig. 2. Immunoaffinity chromatography of hippocampal 5-HT_{1A} receptor. Rat hippocampal 5-HT_{1A} receptor was solubilized and fixed on Affigel-10 immunoaffinity column as described under *Materials and Methods*. Elution with glycine-HCl (pH 2) allowed the desorption of 5-HT_{1A} receptors which could be visualized on immunoblots with specific polyclonal antibody (left lane, Western blot with 5 μl of glycine-HCl eluate). The right lane (Hip, hippocampus) corresponds to 5-HT_{1A} receptor immunolabeling in soluble extract of rat hippocampal membranes (before loading onto the immunoaffinity column). Molecular mass markers are indicated on the left.

5-HT$_{1A}$ receptor-G protein complexes solubilized from hippocampal membranes after incubation with or without 5-CT (10 μM). As shown in Fig. 5, A and B, 5-CT-induced [^{35}S]GTPγS labeling of immunoprecipitates was obtained with anti-Gα_o- and anti-Gα_{i3} antibodies. This effect was mediated by 5-HT$_{1A}$ receptor activation because it was completely inhibited in the presence of WAY 100635. In contrast, immunoprecipitation with anti-Gα_q, anti-Gα_z, and anti-Gα_s yielded no 5-CT-induced increase in [^{35}S]GTPγS labeling of immunoprecipitates adsorbed onto protein A-Sepharose beads (Fig. 5C).

SPA Determination of Gα Proteins Labeled by [^{35}S]GTPγS in Soluble Extracts from 5-CT-Stimulated Hippocampal and Raphe Membranes. Additional experiments were performed using an SPA technology with a protocol derived from that used with protein A-Sepharose beads and adapted to 96-well microplates. The high sensitivity of the detection by SPA led us to perform experiments with membranes from the anterior raphe area in addition to hippocampal membranes. A shown in Fig. 6A, 5-CT induced a robust increase in [^{35}S]GTPγS binding to both Gα_o- and Gα_{i3}-immunoprecipitated samples, corroborating the results obtained with protein A-Sepharose beads. By contrast, no effect of 5-CT could be detected in assays with anti-Gα_s antibodies.

In the anterior raphe area, a significant increase in [^{35}S]GTPγS binding under 5-CT-stimulated compared with basal conditions was measured when immunoprecipitation was made with anti-Gα_{i3} antibodies (Fig. 6B). In contrast, no 5-CT-induced effect could be detected with anti-Gα_o antibodies.

As expected from its mediation through 5-HT$_{1A}$ receptors, the 5-CT-induced increase in [^{35}S]GTPγS labeling of immunoprecipitates with anti-Gα_{i3} and anti Gα_o antibodies was not observed with membranes that had been incubated with both 5-CT and WAY 100635 (Fig. 6, A and B).

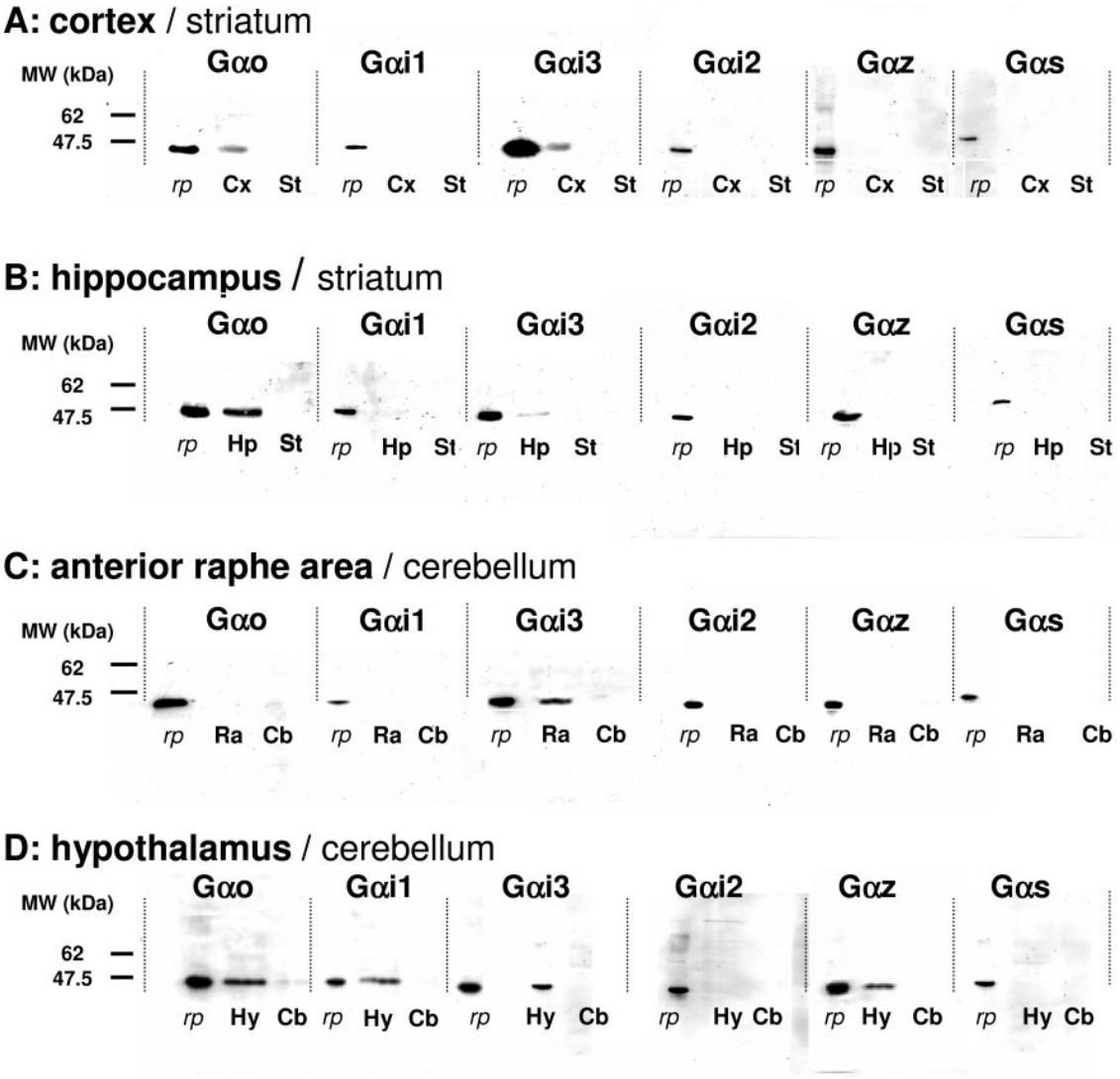

Fig. 3. Immunoblots of different Gα subunits coupled to 5-HT$_{1A}$ receptors in various brain regions. Membrane-bound proteins were solubilized by CHAPS, and 5-HT$_{1A}$ receptor/G protein complexes were adsorbed onto Affigel-10 immunoaffinity column as described under *Materials and Methods*. G proteins were eluted by a 5-HT-GTP mix and identified by immunoblotting with specific anti-Gα antibodies (dilution, 1/200). The *rp* bands correspond to pure recombinant Gα subunits run in parallel with Affigel-10 immunoaffinity column eluates. Note the presence of Gα_o and Gα_{i3} (equally) in the cerebral cortex (A, Cx), of Gα_o (mainly) and Gα_{i3} (trace) in the hippocampus (B, Hip), of Gα_{i3} (exclusively) in the anterior raphe area (C, Ra), and of Gα_o, Gα_{i1}, Gα_{i3}, and Gα_z in the hypothalamus (D, Hy). In contrast, no Gα protein immunolabeling is detected with eluates from striatum (St) or cerebellum (Cb) extracts.

Discussion

Using appropriate immunopurification strategies, we herein report the first direct identification of G proteins coupled to native 5-HT_{1A} receptors in the rat brain. Our data were obtained using two complementary approaches that have been used already for either identifying the coupling between a receptor and a G protein or estimating the efficacy of this coupling (Shreeve, 2002). The immunoaffinity copurification technique relies on the high specificity of the antiserum immobilized on the immunoaffinity column. Previous characterizations have shown that our antiserum selectively immunoprecipitates 5-HT_{1A} but not other 5-HT_1 receptors in solubilized hippocampal membranes and that the receptor/G-protein coupling is preserved in the immune complex (El Mestikawy et al., 1990; Riad et al., 1991). Solubilization of tissue extracts was another critical step, because the deter-

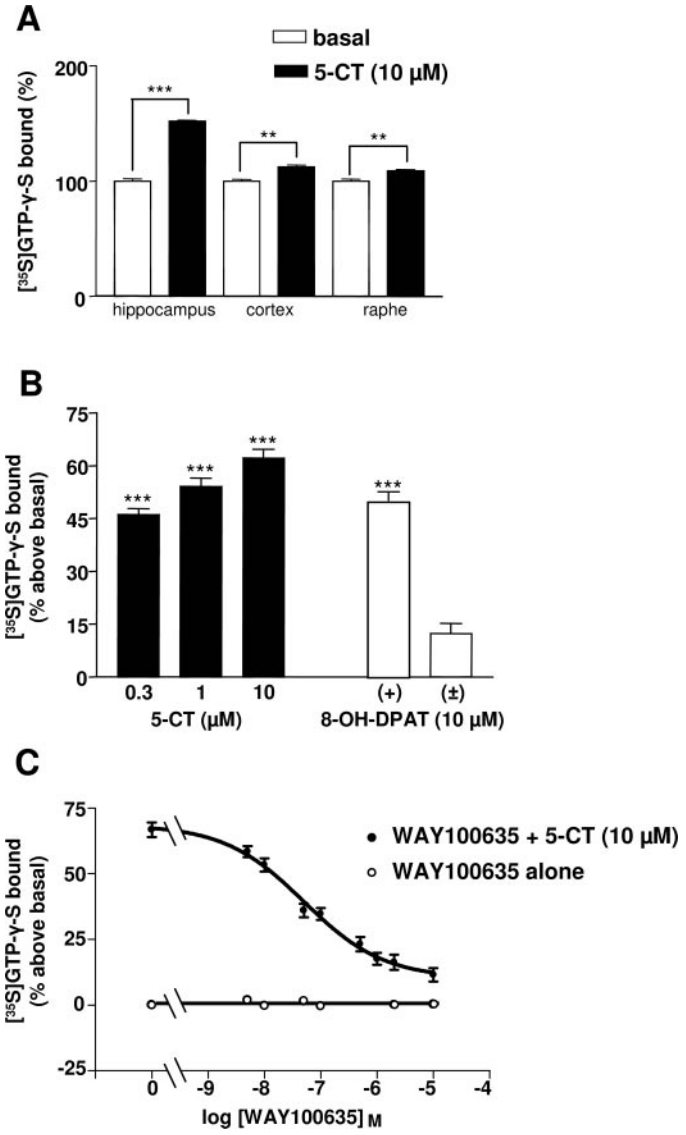

Fig. 4. 5-HT_{1A} receptor-mediated increase in [^{35}S]GTPγS binding in soluble extracts from rat brain membranes. A, 5-CT-stimulated [^{35}S]GTPγS binding in soluble extracts from hippocampus, cerebral cortex, and anterior raphe area membranes. As expected from their lower density of 5-HT_{1A} receptors, the percentage of stimulation above basal was lower in both cerebral cortex and anterior raphe compared with hippocampus. B, differential efficacy of 5-CT and (+) and (±)-8-OH-DPAT to enhance [^{35}S]GTPγS binding in soluble extracts from hippocampal membranes. Each bar corresponds to the percentage increase over basal [^{35}S]GTPγS binding. C, concentration-dependent inhibition by WAY 100635 of 5-CT-stimulated [^{35}S]GTPγS binding in soluble extracts from hippocampal membranes. Each bar/point is the mean ± S.E.M. of triplicate determinations in three different experiments. **, $p < 0.01$; ***, $p < 0.001$ (paired Student's t test).

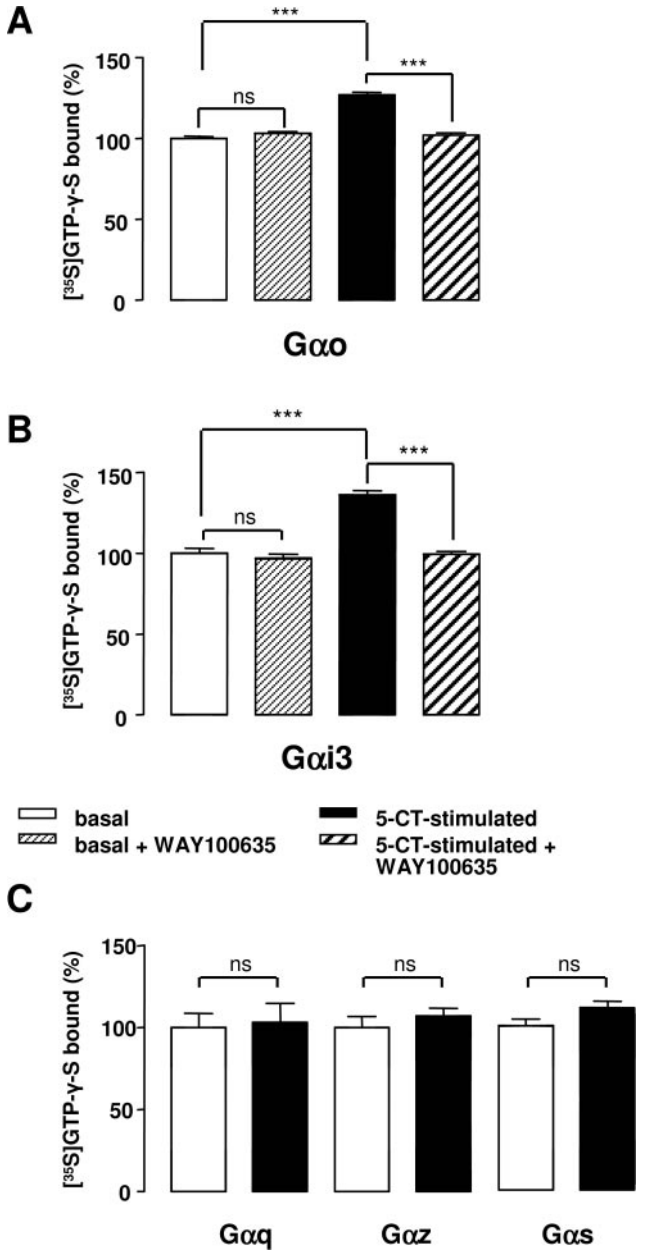

Fig. 5. 5-CT-stimulated [^{35}S]GTPγS binding onto $G\alpha_o$ and $G\alpha_{i3}$ but not $G\alpha_q$, $G\alpha_z$, and $G\alpha_s$ subunits in soluble extracts from rat hippocampal membranes: antibody capture assays. [^{35}S]GTPγS binding onto $G\alpha_o$ (A) and $G\alpha_{i3}$ (B) subunits from 5-HT_{1A} receptor/G protein complexes solubilized from hippocampal membranes after incubation with 10 μM 5-CT and/or 10 μM WAY 100635. ^{35}S-labeled Gα subunits were immunoprecipitated and then bound onto protein A-Sepharose beads as described under *Materials and Methods*. In contrast to anti-$G\alpha_o$ and anti-$G\alpha_{i3}$ antibodies, anti-$G\alpha_q$, -$G\alpha_z$, and -$G\alpha_s$ antibodies (C) did not yield any 5-CT-stimulated ^{35}S-labeling. Results are expressed as the percentage of basal labeling with [^{35}S]GTPγS (100% = 2424 ± 29 and 1410 ± 43 dpm with anti-$G\alpha_o$ and anti-$G\alpha_{i3}$ antibodies, respectively) in the absence of 5-CT and WAY 100635. Each bar is the mean ± S.E.M. of triplicate determinations in eight independent experiments. ***, $p < 0.001$ (paired Student's t test), ns, not significant.

gent could induce the dissociation of the 5-HT$_{1A}$ receptor/G-protein complexes. However, we found previously that CHAPS enables the solubilization of functional 5-HT$_{1A}$ receptors physically coupled to G proteins (El Mestikawy et al., 1988; Emerit et al., 1990).

For our immunopurification protocols, we also used immunoprecipitation procedures whose reliability depends on the specificity of anti-Gα antiserums. This critical point has been evaluated using recombinant Gα proteins. No cross-reactivity was observed with the used antibodies, except for those directed against Gα_{i1} and Gα_{i3} subunits. Because immunoaffinity chromatography experiments showed that 5-HT$_{1A}$ receptors did not couple with Gα_{i1} in the hippocampus and the anterior raphe area, it is probable that the radioactivity measured in immunoprecipitates with anti-Gα_{i3} antibodies resulted from the precipitation of Gα_{i3}- and not Gα_{i1}-[^{35}S]GTPγS-labeled complexes. Concerning the SPA approach, the selectivity of the detection was improved by using secondary antibodies coated on beads that recognize the primary antiserum more specifically than the protein A-Sepharose does. The resulting reduction of the background noise makes this technique more sensitive and enabled the detection of low-intensity signals such as those obtained with raphe membranes (Fig. 6B).

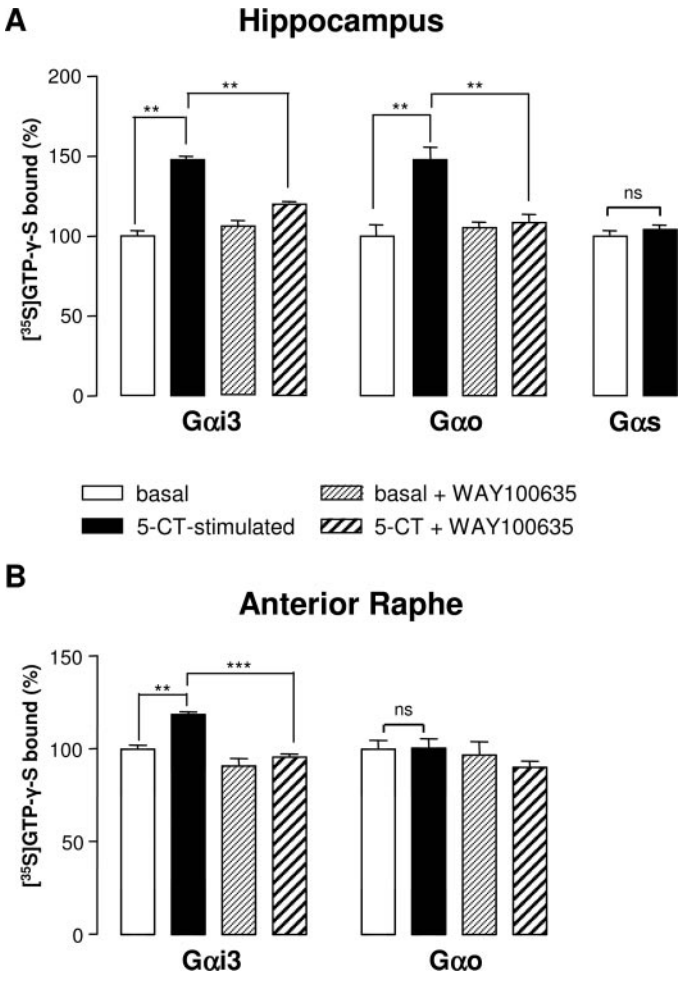

Fig. 6. Differential 5-CT-stimulated [^{35}S]GTPγS binding onto Gα_{i3} and Gα_o in the hippocampus and the anterior raphe area: scintillation proximity assays. [^{35}S]GTPγS binding onto Gα_{i3} and Gα_o subunits was measured using SPA after incubation of hippocampal (A) and anterior raphe (B) membranes with 10 μM 5-CT and/or 10 μM WAY 100635. Under similar conditions with hippocampal membranes, no 5-CT-induced [^{35}S]GTPγS labeling was found with anti-Gα_s antibodies (A). Results are expressed as the percentage of basal values in the absence of 5-CT and WAY 100635 (100%, hippocampus: 1080 ± 75 and 938 ± 28 dpm; anterior raphe area: 586 ± 28 and 636 ± 18 dpm, with anti-Gα_o and anti-Gα_{i3} antibodies, respectively). Each bar is the mean ± S.E.M. of triplicate determinations in four independent experiments. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant; paired Student's t test.

One of the most important observations of our study is that the G-protein coupling of 5-HT$_{1A}$ receptors exhibited clear-cut regional differences in the rat brain. However, all of the G proteins that interact with 5-HT$_{1A}$ receptors in the cortex, hippocampus, hypothalamus, and anterior raphe area belong to the G$_i$/G$_o$ family (Gα_o, Gα_{i1}, Gα_{i3}, and Gα_z). All of them have already been identified using heterologous coexpression of 5-HT$_{1A}$ receptors with various Gα subunits in recombinant systems (Bertin et al., 1992; Raymond et al., 1993; Garnovskaya et al., 1997).

In vitro data have suggested that 5-HT$_{1A}$ receptor/Gα_{i2} coupling results in both AC inhibition and increase of intracellular Ca^{2+} concentration (Raymond et al., 1993; Albert et al., 1996). Despite these data, no band corresponding to Gα_{i2} was identified in any tested fractions, indicating that, in the rat brain, native 5-HT$_{1A}$ receptors do not activate this G-protein subtype.

Although no direct interaction between Gα_s protein and 5-HT$_{1A}$ receptor has been observed in transfected Chinese hamster ovary cells (Raymond et al., 1993), mutations in the third intracellular loop of the 5-HT$_{1A}$ receptor have been shown to induce a weak Gα_s coupling (Malmberg and Strange, 2000). Furthermore, both microdialysis studies and biochemical experiments performed in rat hippocampus demonstrated an increased cAMP formation in response to 5-HT$_{1A}$ receptor agonists, such as 8-OH-DPAT and 5-CT, suggesting a positive AC coupling of 5-HT$_{1A}$ receptors through Gα_s stimulation (Shenker et al., 1987; Cadogan et al., 1994). However, it has to be stressed that the agonists used in these studies can stimulate other 5-HT receptors in addition to the 5-HT$_{1A}$ type, notably 5-HT$_7$ receptors, which are known to activate AC via Gα_s proteins (Hamon, 1997). In fact, our data clearly showed that 5-HT$_{1A}$ receptors interact with Gα_s in neither the hippocampus nor any other brain structures examined. In fact, Albert et al. (1999) found that the 5-HT$_{1A}$ receptor-stimulated production of cAMP in HEK 293 cells requires the coexpression of AC type II, which constitutive activation involves $\beta\gamma$ subunits probably originating from Gα_{i2} proteins. Taken together, these data suggest that a positive coupling between 5-HT$_{1A}$ receptors and Gα_s might occur only under specific conditions that require both specific cellular milieu and particular AC subtypes that are not found in rat brain extracts.

In agreement with previous data in recombinant systems (Bertin et al., 1992; Raymond et al., 1993; Garnovskaya et al., 1997), clear-cut interaction of native 5-HT$_{1A}$ receptors with Gα_{i3} subunit was demonstrated in the cortex, the hypothalamus, the anterior raphe region, and the hippocampus of the rat brain. However, in the hippocampus, immunoaffinity chromatography experiments indicated that 5-HT$_{1A}$ receptors coupled mainly with Gα_o and, to a lower extent, with Gα_{i3}. On the other hand, immunoprecipitation experiments evidenced that 5-CT similarly increased [^{35}S]GTPγS binding onto both G-protein subtypes, suggesting that 5-HT$_{1A}$ receptors could activate Gα_o and Gα_{i3} with the same efficacy. Such

discrepancies might be related to immunoaffinity chromatography conditions, in which only one active state of 5-HT_{1A} receptor could be present and interact essentially with the $G\alpha_o$ subunit. In contrast, in immunoprecipitation experiments, the high-efficacy agonist 5-CT might stimulate two different active states of the receptor coupled to either $G\alpha_o$ or $G\alpha_{i3}$ subunit (Kenakin, 1995). On the other hand, $G\alpha_o$ and $G\alpha_{i3}$ subunits may display a difference in the rate of GDP dissociation, as already shown in the case of $G\alpha_o$ and $G\alpha_{i1}$ (Remmers et al., 1999). Therefore, the $G\alpha_{i3}$-GTP complex would be less stable and $G\alpha_{i3}$ intrinsic GTPase activity higher than that of $G\alpha_o$ subunit. Such characteristics would explain the lower signal obtained with $G\alpha_{i3}$ in immunoaffinity chromatography experiments. Finally, this coupling could also involve specific regulators of G-protein signaling (RGSs). Indeed, differences have been reported between RGS regulating $G\alpha_o$- versus $G\alpha_i$-GTPase activity (Lan et al., 2000).

The coupling of native 5-HT_{1A} receptors to $G\alpha_o$ in the hippocampus corroborates previous results from electrophysiological experiments. Relevant studies demonstrated that 5-HT_{1A} receptor stimulation opens a GIRK conductance through the activation of $G\alpha_{o1}$-protein subtype in hippocampal granule cells (Oleskevich, 1995). It is interesting to note that more recent data from knockout mice evidenced that, in the hippocampus, $G\alpha_o$ is the predominant G protein used for coupling both $GABA_B$ and adenosine receptors to K^+ channels (Greif et al., 2000). This conclusion can probably be extended to 5-HT_{1A} receptors, because the latter have been shown to share the same pool of G proteins with $GABA_B$ and adenosine receptors in the hippocampus (Mannoury la Cour et al., 2004). In contrast, no interaction with $G\alpha_o$ has been detected in the anterior raphe area in which we found that 5-HT_{1A} receptors physically coupled to $G\alpha_{i3}$ only.

This disparity between the hippocampus and the anterior raphe area is particularly relevant regarding the differential regulation of 5-HT_{1A} receptors. Long-term inactivation of 5-HT reuptake by SSRI treatment in rats and 5-HTT gene disruption in 5-HTT−/− mice induce a functional desensitization of 5-HT_{1A} autoreceptors within the DRN but no adaptive changes of 5-HT_{1A} heteroreceptors in the hippocampus (Le Poul et al., 2000; Mannoury la Cour et al., 2001). Transductional modifications are probably at the origin of such regional differences in adaptive regulation of 5-HT_{1A} receptors. Indeed the intronless structure of the 5-HT_{1A} receptor gene is incompatible with the possible existence of several forms of the receptor protein. This desensitization seemed to be associated with a decrease in 5-CT-stimulated [^{35}S]GTPγS binding only in the DRN, suggesting an alteration of receptor/G-protein coupling in this region (Fabre et al., 2000; Hensler, 2002). Our results suggest that desensitization could implicate an alteration of the coupling of 5-HT_{1A} receptors with $G\alpha_{i3}$ but not $G\alpha_o$ subunits. It is interesting that a recent in vivo study indicated that overexpression of RGS4 within the DRN attenuated specific $G\alpha_i$-mediated 5-HT_{1A} receptor signaling, leading to a decrease in 5-HT_{1A} autoreceptor functional response (Beyer et al., 2004). In contrast, such a mechanism would not exist in the hippocampus in which 5-HT_{1A} receptors mediate K^+ channel opening essentially through $G\alpha_o$ subunits (Oleskevich, 1995).

Therefore, in regions in which 5-HT_{1A} receptors are coupled with several G proteins, adaptive compensatory changes might occur to preserve the functional characteristics of the receptors. This might take place in the hippocampus (Greif et al., 2000) and in the cerebral cortex, in which the 5-HT_{1A} receptor/G-protein coupling is unaffected by long-term treatment with fluoxetine (Hensler, 2002). In the hypothalamus, 5-HT_{1A} receptors seemed to be coupled to four different $G\alpha$ subunits, $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_z$. In line with our data, a recent study demonstrated the existence of 5-HT_{1A} receptor-$G\alpha_z$ interaction in the hypothalamic paraventricular nucleus using $G\alpha_z$ antisense oligodeoxynucleotides (Serres et al., 2000). It is interesting that hypothalamic 5-HT_{1A} receptors have also been shown to be functionally desensitized after long-term SSRI administration. This adaptive change was reported to be associated with a reduction in the levels of $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_{i3}$ proteins (Li et al., 1997) and a decrease in membrane-bound $G\alpha_z$ protein (Raap et al., 1999). Such a down-regulation of all $G\alpha$ proteins coupled to hypothalamic 5-HT_{1A} receptors (i.e., the absence of any opposite compensatory changes among these proteins) probably accounts for 5-HT_{1A} receptor desensitization in this particular region.

In conclusion, our data demonstrate that, in the rat brain, regional differences exist regarding the $G\alpha$ protein subtypes that interact with native 5-HT_{1A} receptors. These differences are particularly striking in the anterior raphe area versus the hippocampus, in which differential adaptive changes in 5-HT_{1A} receptors have been reported repeatedly after long-term blockade of 5-HT reuptake. Determinations of 1) $G\alpha_{i3}$ and $G\alpha_o$ mRNA and protein levels, 2) associated $G\beta$ subunits, and 3) the stoichiometry between $G\alpha_{i3}$/$G\alpha_o$ and $G\beta$ subunits, specifically in cells expressing 5-HT_{1A} receptors, will ultimately provide key data concerning the regional differences in 5-HT_{1A} receptor signaling and regulation. In addition, deciphering the mechanisms through which differential coupling occurs is definitively the further goal to be achieved. In particular it will be necessary to identify the different partner proteins that interact with these particular G proteins in the transduction cascade downstream of 5-HT_{1A} receptor stimulation. The probable presence of several RGS proteins in 5-HT_{1A} receptor-expressing cells, the heterogeneity of G protein-coupled receptor kinases, and possible regional differences in 5-HT_{1A} receptor-dependent effectors (such as GIRK channels) has also to be considered in studies aimed at explaining the functional and regulatory heterogeneity of brain 5-HT_{1A} receptors.

Acknowledgments

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