0026-895X/97/040623-09$3.00/0

MOLECULAR PHARMACOLOGY, **52:**623–631 (1997).

5-Hydroxytryptamine$_{1A}$ and 5-Hydroxytryptamine$_{1B}$ Receptors Stimulate [^{35}S]Guanosine-5′-*O*-(3-thio)triphosphate Binding to Rodent Brain Sections as Visualized by *In Vitro* Autoradiography

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Received March 3, 1997; Accepted June 30, 1997

SUMMARY

[^{35}S]Guanosine-5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) binding to G proteins was measured by *in vitro* autoradiography in guinea pig and rat brain sections after activation by 5-hydroxytryptamine (5-HT) receptor agonists. 5-Carboxamidotryptamine stimulated binding strongly in hippocampus and lateral septum and weakly in substantia nigra. This effect was blocked in the substantia nigra by the 5-HT$_{1B/1D}$ receptor antagonist GR-127,935 and in the former two regions by the 5-HT$_{1A}$ antagonist NAN-190. 5-HT$_{1B/1D}$ receptor agonists stimulated binding in substantia nigra and in areas containing 5-HT$_{1A}$ receptors. In guinea pig substantia nigra, 5-(nonyloxy)-tryptamine maximally stimulated [^{35}S]GTPγS binding by 54%, with an EC$_{50}$ value of 62 nM; at 100 μM, this agonist increased binding by ~200% in hippocampus (with a 2-fold weaker EC$_{50}$ value). The distribution of [^{3}H]8-OH-DPAT binding sites was identical to that of the [^{35}S]GTPγS labeling stimulated by the 5-HT$_{1A}$ agonist (*R*)-8-hydroxy-2-dipropylaminotetralin [(*R*)-8-OH-DPAT)]. (*R*)-8-OH-DPAT, (*S*)-8-OH-DPAT, and buspirone stimulated [^{35}S]GTPγS binding in hippocampus by 340%, 140%, and 78%, with EC$_{50}$ values of 71, 51, and 132 nM. Enhanced [^{35}S]GTPγS binding was not detected in the presence of 5-HT$_{1F}$, 5-HT$_{2}$, 5-HT$_{4}$, and 5-HT$_{7}$ receptor agonists. Because activation of μ-opioid, muscarinic M$_2$, histamine H$_3$, and cannabinoid receptors was also visualized successfully, these data suggest that only receptors coupled to pertussis toxin-sensitive G proteins can be seen by [^{35}S]GTPγS binding autoradiography. This study also shows that different 5-HT receptors coupled to these proteins can show a wide range of [^{35}S]GTPγS binding stimulation. Although the functional significance of these variations is unclear, this technique offers advantages over receptor autoradiography because it does not require high affinity radioligands and provides a measure of agonist efficacies in various brain regions.

5-HT exerts a wide variety of actions in the central and peripheral nervous systems by stimulating ≥14 different receptor subtypes (1). With the exception of 5-HT$_3$ receptors, which form an ion channel, all the known subtypes belong to the superfamily of receptors coupled to G proteins. The 5-HT$_1$ class (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, and 5-HT$_{1F}$) is linked to adenylate cyclase inhibition. 5-HT$_2$ receptors (5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$) increase phosphatidylinositide turnover. 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$ receptors stimulate adenylate cyclase. The second messenger system used by 5-HT$_{5A}$ and 5-HT$_{5B}$ receptors is unknown. Although most of this diversity has been demonstrated in recent years by molecular cloning techniques, radioligand binding techniques (both on brain homogenates and tissue sections) have been instrumental in the discovery of the first receptor subtypes. Binding studies are easier and faster than physiological or biochemical (second messenger) experiments. They have, however, two major drawbacks: they are not useful to predict agonist efficacy and they require a high affinity radioligand for the target receptor.

The activation of G proteins by specific receptors has been assayed by measuring [^{35}S]GTPγS binding in isolated membrane preparations (2). This nucleotide is an analogue of GTP, which is exchanged for GDP bound to the α subunit of the G protein after its activation by the agonist/receptor complex. Unlike GTP, [^{35}S]GTPγS cannot be hydrolyzed by the intrinsic GTPase activity of the α subunit, and its incorporation into the membrane can be measured after filtration

This work was supported by National Institutes of Health Grant 1-P01-NS35611–01 (M.A.M.). C.W. is the recipient of a Research Fellowship of the Migraine Trust, and M.A.M. is the recipient of a Bristol-Myers Unrestricted Research Award in Neuroscience.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin; 5-CT, 5-carboxamidotryptamine; GTI, serotonin-5-*O*-carboxymethyl-glycyl-tyrosinamide; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

by liquid scintillation counting. This method addresses successfully the drawbacks mentioned above for radioligand binding studies. It has been used in membrane preparations for several receptors, including adenosine A_1 (3); acetylcholine muscarinic M_2 (4); μ-opioid (5); dopamine D_2 and D_3 (6); metabotropic glutamate$_2$, metabotropic glutamate$_4$, and metabotropic glutamate$_6$ (7, 8); and 5-HT_{1A} (9), 5-HT_{1B}, and 5-HT_{1D} (10) receptors. In all these reports, agonist-stimulated binding of [^{35}S]GTPγS could be observed only in the presence of 1–10 μM GDP, which seemed to be required to keep G proteins in their GDP-liganded form. Recently, Sim *et al.* (11) adapted this technique (essentially by increasing the GDP concentration to 2 mM) to autoradiographically visualize [^{35}S]GTPγS binding in brain sections after activation of μ-opioid, cannabinoid, and γ-aminobutyric acid$_B$ receptors.

We now report the use of this autoradiographic approach to study the regional pattern of receptor-stimulated [^{35}S]GTPγS binding in guinea pig and rat brain using drugs active at different 5-HT receptor subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_4, and 5-HT_7). We also investigated the pharmacological profile of these responses and analyzed the distribution of [^{35}S]GTPγS labeling at the light microscopic level. [Part of this work has been presented previously in abstract form (12)].

Experimental Procedures

Materials. [^{3}H]5-CT and [^{35}S]GTPγS were obtained from New England Nuclear Research Products (Boston, MA) (specific activity, 22.8 and 1000–1500 Ci/mmol, respectively). [^{3}H]8-OH-DPAT was obtained from Amersham (Arlington Heights, IL) (specific activity, 205 Ci/mmol). GR-127,935 (2′-methyl-4′-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]amide), naratriptan (*N*-methyl-2-[3,1-methylpiperidin-4-yl)-1*H*-indol-5-yl]ethanesulfonamide), and sumatriptan were provided by Glaxo. CP-122,288 [5-methylaminosulfonylmethyl-3-(*N*-methylpyrrolidin-2*R*-ylmethyl)-1*H*-indole] was obtained from Pfizer (Groton, CT). GTI was obtained from Immunotech (Marseilles, France). GTPγS and GDP were purchased from Sigma Chemical (St. Louis, MO). All other drugs were obtained from Research Biochemicals (Natick, MA).

Tissues. Adult male rats (Sprague-Dawley, 200–250 g) and adult guinea-pigs (Hartley, 250–350 g) were sedated with inhaled chloroform and decapitated. The whole brain and upper cervical spinal cord was dissected and frozen in isopentane cooled at −40°. Frozen brains were sectioned using a cryostat-microtome (1720; Leica, Deerfield, IL). The sections were thaw-mounted onto gelatinized glass slides and stored at −80° for <1 month. Preliminary experiments using 8-, 14-, and 20-μm-thick sections showed that 10 μM 5-CT increased [^{35}S]GTPγS binding by 1050 ± 185% (mean ± standard error), 680 ± 120%, and 450 ± 55%, respectively, in guinea pig hippocampus; therefore, 10-μm-thick sections were used in subsequent experiments.

Autoradiography. [^{35}S]GTPγS binding was visualized using the method developed by Sim *et al.* (11), with minor modifications. Briefly, the tissue sections (from at least five different animals) were brought to room temperature 15 min before the experiment; incubated for 15 min at room temperature in 50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 0.2 mM dithiothreitol; and then incubated for an additional 15 min in the same buffer supplemented with 2 mM GDP. Agonist-stimulated binding was determined by incubating the sections for 60 min at 30° in buffer containing 2 mM GDP, 0.04 nM [^{35}S]GTPγS, and the appropriate concentration of agonist and/or antagonist. Nonspecific binding was assessed by including 10 μM unlabeled GTPγS in the incubation buffer. Slides were then washed twice for 3 min in ice-cold 50 mM HEPES buffer, pH 7.0, dipped briefly in ice cold distilled water, dried under a stream of cold air, and exposed to Hyperfilm βmax (Amersham) for 24 hr. All experiments were performed independently at least twice.

[^{3}H]5-CT and [^{3}H]8-OH-DPAT binding sites were labeled as described previously (13) using 1 nM concentration of either ligand. Exposure times (to Hyperfilm ^{3}H) were 3 weeks for [^{3}H]8-OH-DPAT and 6 weeks for [^{3}H]5-CT.

Light microscopic autoradiography. After exposure to Hyperfilm βmax films, selected slides were coated with Kodak NTB2 liquid emulsion (diluted 1:1 with water and maintained at 40°), allowed to dry in a humid chamber, and kept at 4° for 2 weeks in boxes containing Silicagel. They were developed in Kodak D-19 (diluted 1:1 with water) for 3 min at 16° and fixed with Kodak Polymax fixer (diluted 1:8 with water). The sections then were stained with 1% basic fuchsin for 10 sec and coverslips were affixed.

Image analysis. The absorbance of the autoradiograms was measured over selected brain regions using a computerized image analysis system (M4; Imaging Research, St. Catherines, Ontario, Canada). [^{35}S]GTPγS autoradiograms were analyzed by comparing the absorbance of the films with the absorbance of a Kodak calibrated density step tablet. Because parallel experiments with ^{14}C standards indicated that the maximal absorbances observed on [^{35}S]GTPγS autoradiograms were still within the linear domain of the film exposure-response curve (except for WIN 55212–2-stimulated binding in globus pallidus and substantia nigra), radioactive standards were not routinely used. Agonist-induced [^{35}S]GTPγS binding is expressed as percentage of basal binding.

Data analysis. Data points from autoradiographic measurements were fitted by nonlinear regression using Grafit (Erithacus Software, Staines, UK). The equation used was $\text{Stim} = E_{max}/(1 + EC_{50}/\text{Ago})$, where Stim is the stimulated binding (percent over basal), E_{max} is the maximal binding, EC_{50} is the concentration of agonist resulting in half-maximal [^{35}S]GTPγS binding, and Ago is the agonist concentration. The pK_B values for antagonists (NAN-190 at 5-HT_{1A} receptors) were calculated from the rightward shift of agonist concentration-response curves according to the formula $pK_B = \log(CR - 1) - \log(\text{Anta})$, where CR is the ratio of agonist IC_{50} values with or without antagonist, and Anta is the antagonist concentration.

Results

Unless otherwise stated, the following results refer to both guinea pig and rat brains.

Basal [^{35}S]GTPγS labeling. In the absence of GDP in the incubation buffer, basal [^{35}S]GTPγS labeling was homogeneously very high throughout the brain, and no increase in binding was detected in the presence of 10 μM 5-CT. As reported previously (11), optimal agonist stimulation was observed when 2 mM GDP was included. No improvement was found with 4 mM GDP, and the stimulation disappeared at 8 mM GDP (not shown). All subsequent experiments were thus carried out with 2 mM GDP. The basal [^{35}S]GTPγS observed under these conditions was likely to be specific because the labeling was not different from film background when 10 μM unlabeled GTPγS was included in the buffer. Basal binding (i.e., in the absence of added agonist) was regionally heterogeneous (Figs. 1A and 2A). The highest level was found in the substantia gelatinosa of the spinal cord and medulla, followed by the interpeduncular nucleus and substantia nigra; intermediate levels were also found in hippocampus, central gray, and superficial layer of the superior colliculus. The cortex and striatum bound only slightly more [^{35}S]GTPγS than white matter areas (which contain very low but GTPγS-displaceable labeling). The level of basal binding,

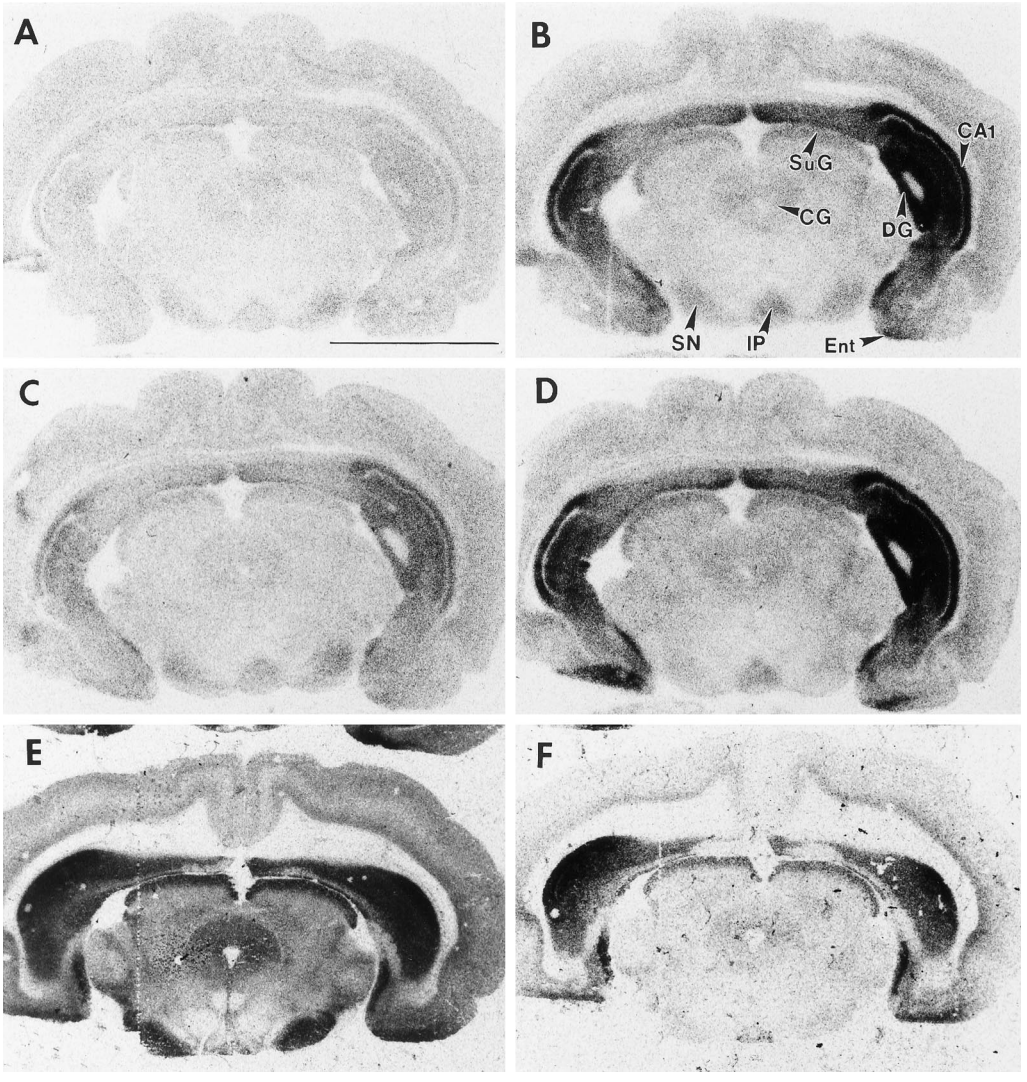

Fig. 1. Distribution of [^{35}S]GTPγS and [^{3}H]5-CT binding sites in guinea pig brain at the level of posterior hippocampus (similar patterns were observed in four other animals in two independent experiments). A, Basal [^{35}S]GTPγS binding is low, but heterogeneous, with higher levels in substantia nigra (*SN*), hippocampus [dentate gyrus (*DG*); *CA1*, subfield of Ammon's horn], interpeduncular nucleus (*IP*), superior colliculus (*SuG*), and central gray (*CG*). B, 5-CT (10 μM) markedly increases [^{35}S]GTPγS binding in hippocampus, superior colliculus, and entorhinal cortex (*Ent*) but does so only weakly in substantia nigra. The increase in the latter region is blocked by the 5-HT$_{1B/1D}$ antagonist GR-127,935 (10 μM; D); in the former three regions, it is blocked by the 5-HT$_{1A}$ antagonist NAN-190 (10 μM; C). The labeling pattern obtained using [^{3}H]5-CT as a ligand is shown (E); comparably high densities of binding sites are seen in hippocampus, superior colliculus, and substantia nigra. Intermediate densities of sites are also found in the superficial and deep cortical layers. The addition of blockers of the 5-HT$_{1D}$ (10 μM GR-127, 935) and 5-HT$_7$ (1 μM spiperone) receptor subtypes does not affect 5-HT$_{1A}$ sites (F), concentrated in hippocampus, superior colliculus, and deep cortical layers. Note that 10 μM 5-CT increases [^{35}S]GTPγS binding in these deep layers but not in the superficial layers (B), which are known to contain 5-HT$_7$ receptors. *Scale bar*, 5 mm.

in particular in the medulla, was not decreased when the animals were killed with pentobarbital overdose (no decapitation), when the sections were subjected to a longer preincubation (even in the presence of 1 mM GTP to accelerate the dissociation of endogenous ligands), or by increasing the washing time up to 15 min (results not shown). None of the antagonists used in the study (NAN-190, GR-127,935, or methiothepine) produced a detectable reduction of basal labeling.

5-CT stimulated [^{35}S]GTPγS labeling. The potent, but nonselective, 5-HT receptor agonist 5-CT (10 μM) increased [^{35}S]GTPγS binding very strongly in the hippocampal formation and lateral septum (latter area not shown) but only weakly in the superficial gray layer of the superior colliculus, central gray, interpeduncular nucleus, substantia gelatinosa of the medulla (trigeminal nucleus caudalis; not shown), neocortex, substantia nigra, and globus pallidus (not shown) (Fig. 1). The selective 5-HT$_{1B/1D}$ receptor antagonist GR-127,935 (10 μM) inhibited this effect only in substantia nigra and globus pallidus, whereas the selective 5-HT$_{1A}$ receptor antagonist NAN-190 (10 μM) was effective in the other areas. A strikingly different labeling pattern was found when [^{3}H]5-CT was used as a radioligand. It labeled equally high densities of sites in hippocampus, substantia nigra, and superior colliculus. Intermediate densities of [^{3}H]5-CT binding sites [known to correspond to 5-HT$_7$ receptors (13)] were also observed in the superficial cortical layers. Fig. 1F shows the labeling pattern obtained after blockade of [^{3}H]5-CT binding to 5-HT$_{1D}$ (with 100 nM GR-127,935) and 5-HT$_7$ receptors (with 1 μM spiperone); it is mostly accounted for by 5-HT$_{1A}$ receptors.

5-HT$_{1B/1D}$ receptor stimulated [^{35}S]GTPγS labeling. The effect of a series of agonists with high affinity for 5-HT$_{1B/1D}$ receptors was examined in guinea pig brain (Fig. 2). At 1 μM, L-694,247 (Fig. 2B), 5-(nonyloxy)-tryptamine (Fig. 2C), naratriptan (Fig. 2D), GTI (Fig. 2E), and sumatriptan (not shown) all stimulated [^{35}S]GTPγS binding in the substantia nigra. Varying degrees of stimulation were also observed in regions known to contain 5-HT$_{1A}$ receptors (hippocampus, lateral septum, and superior colliculus). This effect was particularly strong with 10 μM L-694,247, which increased binding in the latter areas to a higher level than in the substantia nigra. The stimulation by 10 μM GTI in hippocampus, lateral septum, and superior colliculus (but not substantia nigra) was abolished in the presence of 10 μM NAN-190 (a 5-HT$_{1A}$ receptor antagonist; Fig. 2F). In the absence of agonist, the 5-HT$_{1B/1D}$ receptor antagonist

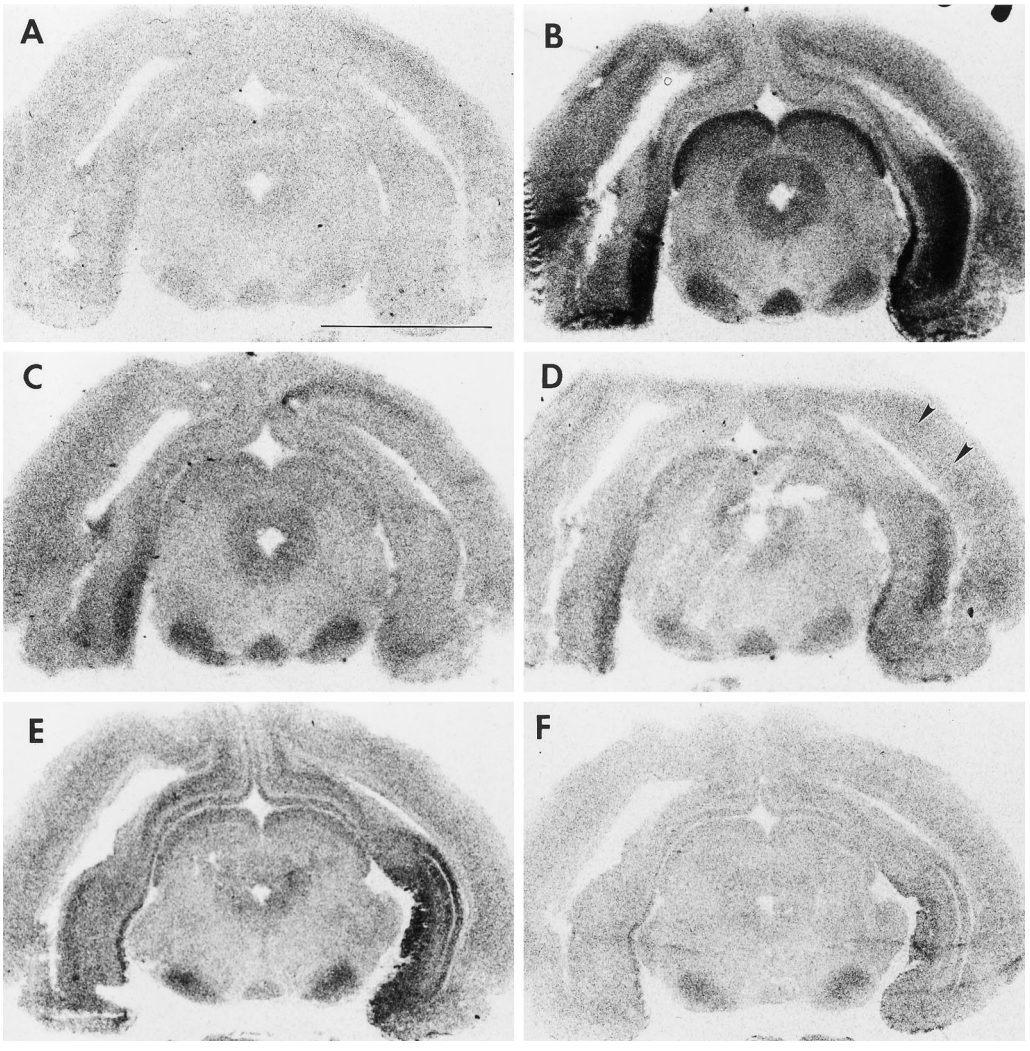

Fig. 2. Effect of several 5-$HT_{1B/1D}$ receptor agonists on [^{35}S]GTPγS binding in guinea pig brain at the level of posterior hippocampus (similar patterns were observed in four other animals in two independent experiments). A, Basal [^{35}S]GTPγS binding. Labeling patterns observed in the presence of 1 μM L-694,247 (B), 5-(nonyloxy)-tryptamine (C), naratriptan (D), and GTI (E). Enhanced binding is found not only in substantia nigra (containing 5-HT_{1D} receptors) but also in hippocampus (containing 5-HT_{1A} receptors). In the presence of 10 μM NAN-190 (F), GTI-enhanced [^{35}S]GTPγS binding disappears in hippocampus and superior colliculus. The binding remaining in substantia nigra is significantly higher than basal binding (*t* test, $p < 0.01$). Note that naratriptan (D), despite its high potency at 5-HT_{1F} receptors, does not increase binding in the intermediate cortical layers (*arrowheads*), known to contain high densities of 5-HT_{1F} receptors. *Scale bar*, 5 mm.

GR-127,935 ($\leq$10 μM) had no effect on [^{35}S]GTPγS binding in guinea pig substantia nigra (not shown).

Because 5-(nonyloxy)-tryptamine produced a measurable stimulation in substantia nigra with limited effects in hippocampus, this agonist was selected to examine quantitatively the dose-response relationship of 5-$HT_{1B/1D}$ receptor-induced [^{35}S]GTPγS binding (Fig. 3; results shown are representative of two independent experiments). In guinea pig substantia nigra, 5-(nonyloxy)-tryptamine stimulated [^{35}S]GTPγS binding by a maximum of 54 $\pm$ 5%, with an EC_{50} value of 62 $\pm$ 27 nM. In rat substantia nigra, the maximal effect was significantly higher (94 $\pm$ 6%), with a comparable EC_{50} value (117 $\pm$ 36 nM). 5-(Nonyloxy)-tryptamine stimulated [^{35}S]GTPγS binding in hippocampus to a higher extent than in substantia nigra. In rat and guinea pig, 100 μM concentration of this agonist stimulated binding by 107 $\pm$ 11% and 190 $\pm$ 50%, respectively. The maximal effect was apparently not reached at this concentration and the EC_{50} values were not calculated; they were probably $\geq$2 orders of magnitude higher than those observed in the substantia nigra.

5-HT_{1A} receptor stimulated [^{35}S]GTPγS labeling in guinea pig brain. Fig. 4 shows the regional distribution of [^{35}S]GTPγS binding stimulated by 10 μM (*R*)-8-OH-DPAT (Fig. 4, A–D) compared with the distribution of [^{3}H]8-OH-DPAT binding sites at similar levels of the guinea pig brain (Fig. 4, A′–D′). Both techniques reveal a virtually identical distribution of recognition sites. This agreement is particularly noticeable in the hippocampal formation, in which both [^{35}S]GTPγS- and [^{3}H]8-OH-DPAT-labeled sites are concentrated in the molecular layer of the dentate gyrus and in strata oriens and radiatum of Ammon's horn. Much lower densities of bound tracers were seen in the polymorph and granule cell layers of the dentate gyrus and in the pyramidal cell layer of Ammon's horn, indicating that they were present mainly in the dendritic fields of pyramidal and granule cells (see discussion of light microscopy).

Three agonists [(*R*)-8-OH-DPAT, (*S*)-8-OH-DPAT, and buspirone], which are known to have high, intermediate, and low intrinsic activity, respectively, at 5-HT_{1A} receptors, were used to generate dose-response curves (Fig. 5). Their EC_{50} and E_{max} values in hippocampus were 71 $\pm$ 28 nM and 340 $\pm$ 100%, 51 $\pm$ 5 nM and 140 $\pm$ 36%, and 132 $\pm$ 31 nM and 78 $\pm$ 19%, respectively (mean $\pm$ standard error of three independent experiments). The 5-HT_{1A} receptor antagonist NAN-190 did not increase [^{35}S]GTPγS binding at $\leq$10 μM; thus, this antagonist was used at three concentrations (10, 30, and 100 nM) to produce a rightward shift in the dose-response curve of (*R*)-8-OH-DPAT in several brain areas (Fig. 6). In hippocampus, lateral septum, raphe dorsalis, and superior colliculus,

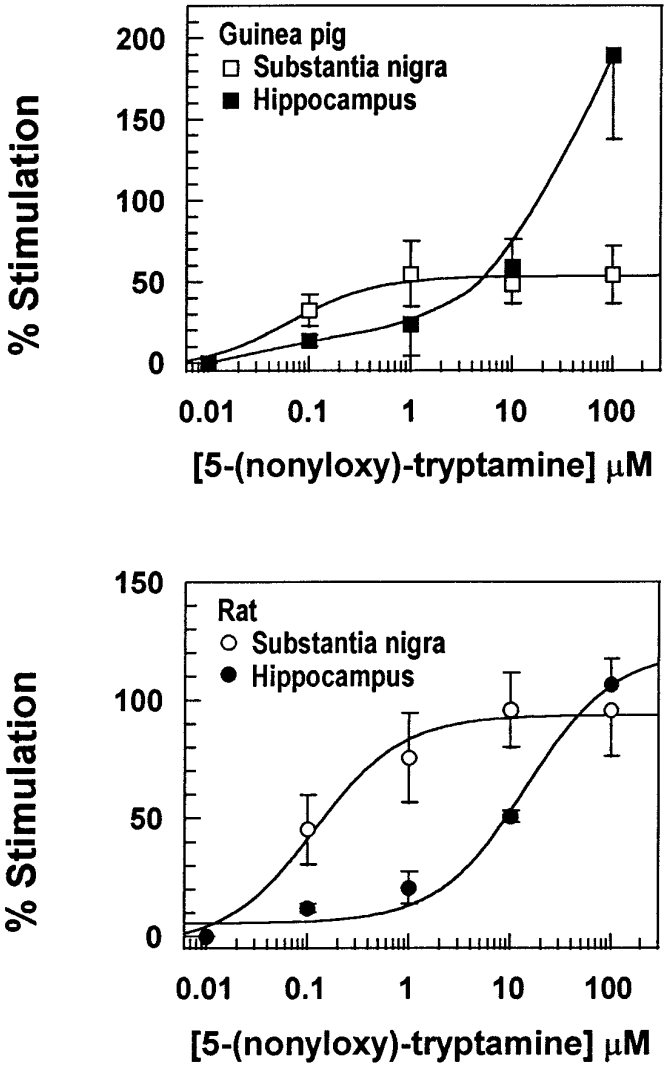

Fig. 3. Effect of increasing concentrations of the 5-$HT_{1B/1D}$ receptor agonist 5-(nonyloxy)-tryptamine on [^{35}S]GTPγS binding in substantia nigra (*open symbols*) and hippocampus (*closed symbols*) of guinea pig (*squares*) and rat (*circles*). Data are expressed as percent increased [^{35}S]GTPγS binding over basal binding (*error bars*, mean ± standard error values observed between different animals in an individual experiment) and are representative of two independent experiments. The agonist shows comparable potencies in both species and is less potent in hippocampus (● and ■) than in substantia nigra (○ and □). Maximal stimulations of [^{35}S]GTPγS binding are 54%, 94%, and 120% in guinea pig substantia nigra, rat substantia nigra, and rat hippocampus, respectively. At 100 μM, 5-(nonyloxy)-tryptamine increases [^{35}S]GTPγS binding by 190% in guinea pig hippocampus.

the calculated K_B values of NAN-190 were 1.7 ± 0.4, 2.2 ± 0.8, 0.5 ± 0.3, and 3.3 ± 1.5 nM.

Effect of 5-HT_{1F}, 5-HT_2, 5-HT_4, and 5-HT_7 receptor agonists on [^{35}S]GTPγS labeling. Both 1 μM (see above) and 30 μM naratriptan (not shown) or 10 μM CP-122,288 (not shown) increased [^{35}S]GTPγS labeling in the substantia nigra (5-$HT_{1B/1D}$ receptors) and hippocampus (5-HT_{1A} receptors). However, in the presence of 10 μM concentration of both GR-127,935 and NAN-190, no enhanced [^{35}S]GTPγS binding was observed with these compounds [in particular, not in the claustrum and neocortex, in which high densities of 5-HT_{1F} receptors have been reported (14)], despite the fact that both drugs possess nanomolar affinity for 5-HT_{1F} sites (10,14). [^{3}H]5-CT has been shown to label high densities of 5-HT_7 sites in the superficial cortical layers and midline thalamic nuclei (13); however, no 5-CT-enhanced [^{35}S]GTPγS labeling was detected in these areas (≤10 μM 5-CT). Finally, neither the 5-$HT_{2A/2C}$ agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane nor the 5-HT_4 agonist SC 53116 (4-amino-5-chloro-*N*-[(hexahydro-1*H*-pyrrolizin-1-yl)methyl]2-methoxybenzamide) was able to increase [^{35}S]GTPγS binding in any brain area (both agonists were used at a concentration of 10 μM).

Species differences in agonist-induced [^{35}S]GTPγS labeling. As mentioned above, 5-(nonyloxy)-tryptamine stimulated [^{35}S]GTPγS binding more strongly in rat substantia nigra (as well as in mouse substantia nigra; not shown) than in guinea pig substantia nigra. The stimulation induced by agonists for other receptor classes (≤10 μM) was compared in rats and guinea pig (Fig. 7). In guinea pig, the [^{35}S]GTPγS labeling in the presence of the μ-opiate agonist DAMGO or muscarinic M_2 agonist oxotremorine barely differed from the basal labeling (with the exception of the superficial gray layer of the superior colliculus and midline thalamic nuclei, in which binding was enhanced strongly by oxotremorine and the mammillary nuclei, densely labeled in the presence of DAMGO). In contrast, both agonists produced a marked and heterogeneous increase in [^{35}S]GTPγS binding in various rat brain areas. DAMGO-enhanced labeling was observed in striatal patches (probably corresponding to striosomes), globus pallidus, several thalamic nuclei, and substantia nigra pars compacta. Oxotremorine increased binding in the rat striatum and superficial gray layer of the superior colliculus. The histamine H_3 receptor agonist imetit stimulated binding weakly in guinea pig striatum, globus pallidus, and substantia nigra; a similar pattern was found in rats but with higher densities of bound [^{35}S]GTPγS. Only stimulation by the cannabinoid agonist WIN55,212–2 resulted in comparable [^{35}S]GTPγS binding pattern in both species, with very dense labeling observed in the globus pallidus and substantia nigra, as well as, to a lesser extent, the hippocampus and cortex.

Light microscopic autoradiography. Fig. 8 shows the distribution of silver grains over different guinea pig brain areas labeled with [^{35}S]GTPγS (dark field microscopy). In the absence of agonist (Fig. 8B), virtually no autoradiographic grains were found over the pyramidal cell layer of Ammon's horn (*CA1*) and the granular cell layer of dentate gyrus and only a low level of diffuse labeling was seen over the surrounding layers. In the presence of 1 μM 8-OH-DPAT (Fig. 8C), no increase in labeling was observed over the pyramidal and granular cell layers, and only a moderate increase was found in the polymorph layer of the dentate gyrus. In contrast, labeling was increased markedly in the strata oriens and radiatum of Ammon's horn and the molecular layer of dentate gyrus. In the superficial gray layer of the superior colliculus, 1 μM 8-OH-DPAT (Fig. 8D) seemed to increase binding homogeneously and mostly in the neuropil, whereas only few grains were observed directly over the cells. A similar distribution was observed in the globus pallidus (Fig. 8E) and substantia nigra reticulata (Fig. 8F) in the presence of a cannabinoid agonist, 1 μM WIN55,212–2.

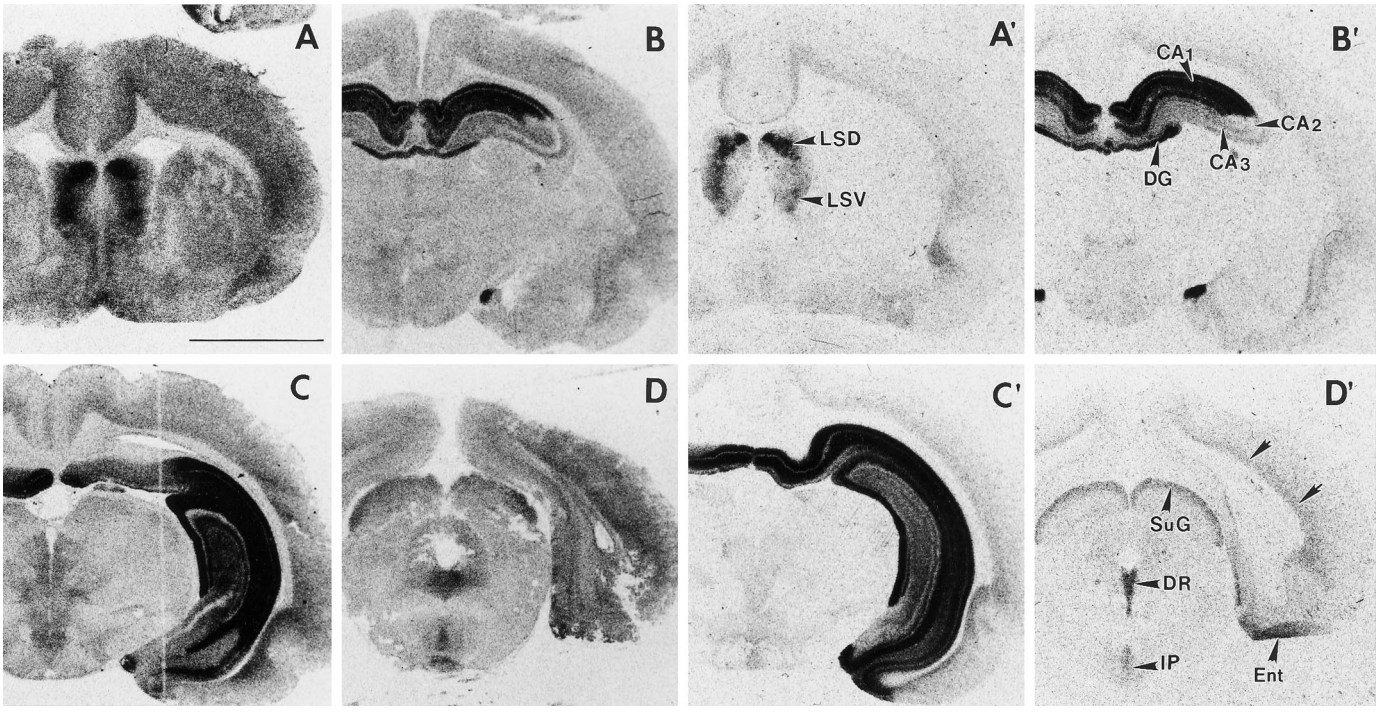

Fig. 4. Distribution of [^{3}H]8-OH-DPAT binding sites (A′–D′) and [^{35}S]GTPγS binding stimulated by 10 μM (+)-8-OH-DPAT (A–D) at several levels of guinea pig brain (similar patterns were observed in four other animals in two independent experiments). The labeling pattern of both ligands showed an excellent correlation in all brain regions investigated. Basal [^{35}S]GTPγS labeling (not shown on this figure) was similar to that displayed in Figs. 1A, 2A, and 7A. Nonspecific [^{3}H]8-OH-DPAT labeling could not be distinguished from film background. High densities of binding sites for both radioligands were observed in the lateral septum [dorsal and ventral nuclei (*LSD and LSV*)], hippocampal formation [particularly in the CA1 subfield and dentate gyrus (*DG*)], superficial gray layer of the superior colliculus (*SuG*), dorsal raphe nucleus (*DR*), interpeduncular nucleus (*IP*), and entorhinal cortex (*Ent*), as well as in the deepest cortical layers (*arrowheads*). The laminar and subfield distributions of [^{35}S]GTPγS and [^{3}H]8-OH-DPAT binding sites in hippocampus overlap completely. *Scale bars,* 5 mm.

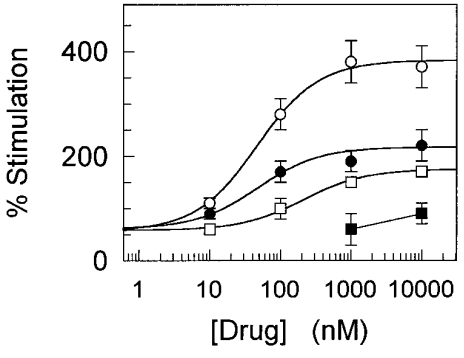

Fig. 5. Effect of increasing concentrations of three 5-HT$_{1A}$ receptor agonists known to possess various intrinsic activities on [^{35}S]GTPγS binding in guinea pig hippocampus. Data are expressed as percent increased [^{35}S]GTPγS binding over basal binding (*error bars,* mean ± standard error values observed in an individual experiment) and are representative of three independent experiments. ○, (*R*)-(+)-8-OH-DPAT; ●, (*S*)-(−)-8-OH-DPAT; □, buspirone data points. ■, 5-HT$_{1A}$ receptor antagonist NAN-190 has no effect of [^{35}S]GTPγS binding (≤10 μM).

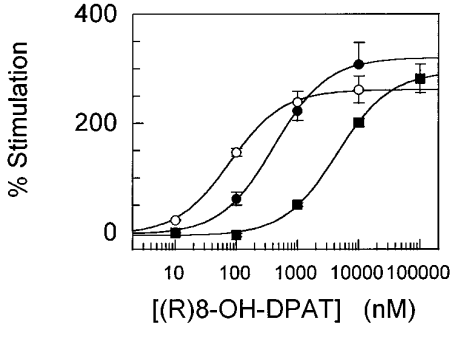

Fig. 6. Effect of increasing concentrations of the 5-HT$_{1A}$ receptor antagonist NAN-190 on the dose-response of (*R*)-(+)-8-OH-DPAT on [^{35}S]GTPγS binding in guinea pig hippocampus. NAN-190 (●, 10 nM; ■, 100 nM) produced a parallel rightward shift in the (*R*)-(+)-8-OH-DPAT dose-response curve (○, absence of antagonist). Similar effects were observed in the lateral septum, raphe dorsalis, and superior colliculus (not shown).

Discussion

5-HT is known to interact with ≥14 different receptor subtypes, of which 13 are coupled to G proteins (1). Taken together, the drugs used in the current study have a high affinity for all these receptors, with the exception of 5-HT$_{1E}$, 5-HT$_{2B}$, and 5-HT$_6$ sites. G protein activation by only two (5-HT$_{1A}$ and 5-HT$_{1B}$) of the remaining 10 receptors was detected using [^{35}S]GTPγS autoradiography. None of the drugs used in this study discriminate between 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors; however, in consideration of the predominance of 5-HT$_{1B}$ receptors in the mammalian brain (15,16), it is likely that these sites account for the enhancement of [^{35}S]GTPγS binding by 5-HT$_{1B}$/5-HT$_{1D}$ agonists.

The failure to detect G protein activation by 5-HT$_{2A/C}$ and 5-HT$_4$ receptors probably reflects the fact that these receptors are coupled to pertussis toxin-insensitive G proteins, and receptors coupled to these classes of G protein have not been reported to stimulate [^{35}S]GTPγS binding in brain membrane preparations. In contrast, there are numerous accounts of enhanced [^{35}S]GTPγS binding induced by G_i- or G_o-coupled receptors (see Introduction). Recently, this tech-

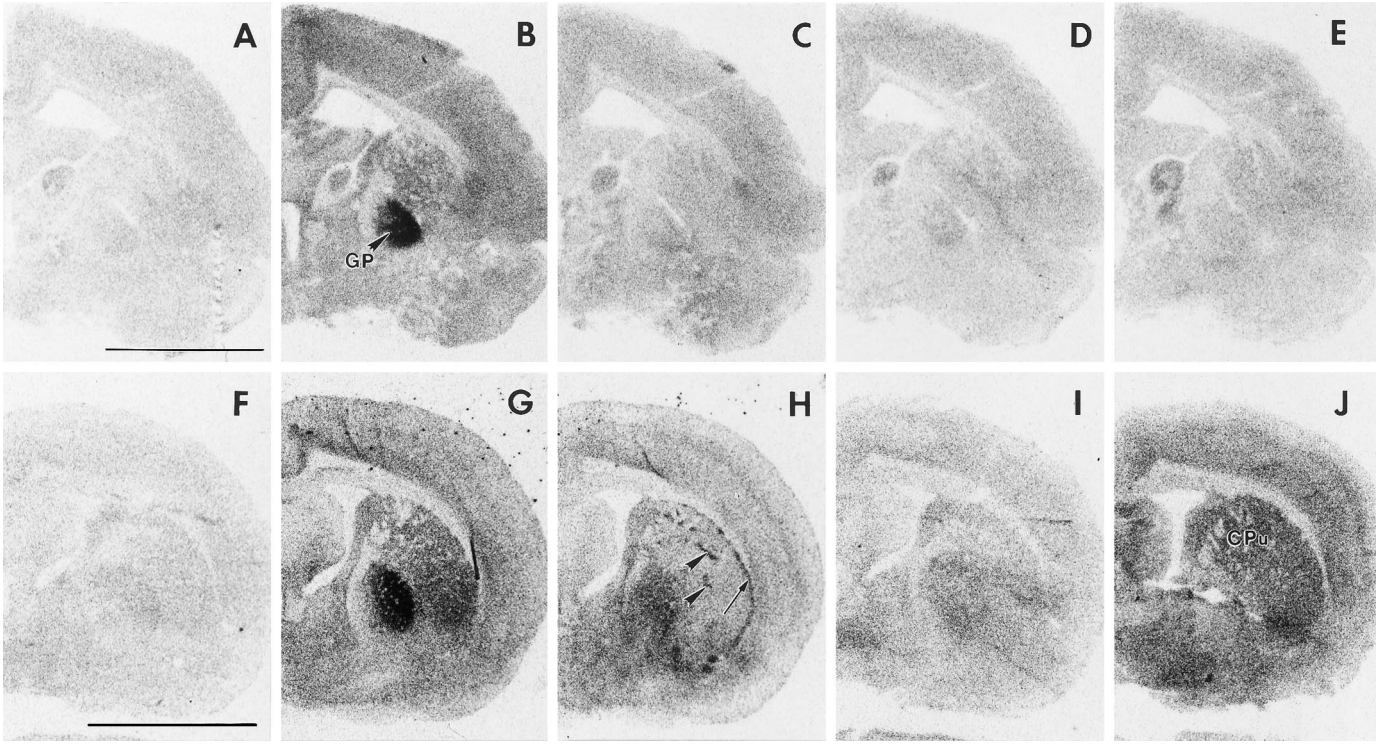

Fig. 7. Effect of agonists for several $G_{i/o}$ protein-coupled receptors on [^{35}S]GTPγS binding in guinea pig (A–E) and rat (F–J) brain (similar patterns were observed in four other animals of each species in two independent experiments). Basal [^{35}S]GTPγS labeling patterns are similar in both species, as are the labeling obtained in the presence of the cannabinoid agonist 10 μM WIN55,212–2 (B and G). However, stimulation of μ-opioid, histamine H_3, and muscarinic M_2 receptors with 10 μM DAMGO (C and H), imetit (D and I), or oxotremorine (E and J), respectively, results in only weak stimulation of [^{35}S]GTPγS binding in guinea pig brain, whereas marked and heterogeneous changes are observed in rat brain. DAMGO-enhanced labeling is prominent in rat globus pallidus (*GP*), striatal patches [probably corresponding to striosomes (*arrowheads*)], and a striatal subcallosal streak (*arrow*) typically observed with μ-opiate receptor radioligands. Imetit and oxotremorine increase [^{35}S]GTPγS binding in globus pallidus and caudate-putamen (*CPu*), respectively. *Scale bars*, 5 mm.

nique was adapted and used on brain sections to visualize the activation of cannabinoid, γ-aminobutyric acid $_B$, and μ-opioid (11), opioid receptor-like (17), δ-opioid (18), and somatostatin (19) receptors. The results of the current report add muscarinic M_2 and histamine H_3 receptors to the list of $G_{o/i}$-coupled receptors activating [^{35}S]GTPγS binding in brain sections.

Several properties might account for the indirect labeling of receptors coupled to pertussis toxin-sensitive, but not other, G proteins (20). First, G_o and G_i are the major G proteins in the brain. Second, activation of the α subunit of G_s requires much higher (25–50 mM) Mg^{2+} concentrations than activation of G_i or G_q α subunits (3 mM Mg^{2+} was used in this and previous studies). Finally, α subunits of different classes show various rates of spontaneous GDP dissociation (2). At 30°, G_i proteins show a rapid spontaneous dissociation of GDP from their α subunits; excess GDP must be added to shift the equilibrium toward the α subunit/GDP complex and allow the detection of agonist-enhanced [^{35}S]GTPγS binding. Alternatively, the reaction can be carried out at 4° but in the absence of added GDP and NaCl (2) (which probably uncouples G proteins from unoccupied receptors). The latter approach has not been used in brain sections, although it might be an interesting alternative to the approach used in this and previous studies. In addition to being relatively expensive, the large amounts of GDP added in the incubation medium might be responsible for the low potencies of agonists observed with this method. Indeed, differences in GDP concentrations might account for the lower EC_{50} value of 8-OH-DPAT in our system (50–70 nM) compared with that obtained on membrane preparations in the presence of only 3 μM GDP (6 nM) (9).

At variance with G_i proteins, G_s proteins show a relatively slow spontaneous dissociation of GDP, and the addition of GDP or NaCl only decreases isoproterenol-induced [^{35}S]GTPγS binding to turkey erythrocyte membranes (2). Several strategies have been proposed to reduce agonist-independent [^{35}S]GTPγS binding on these membranes (2); their use on cryostat brain sections might permit detection of activated receptors coupled to G proteins other than G_i or G_o.

Because 5-HT_{1F} receptors have been reported to inhibit adenylate cyclase in transfected cells (21), the lack of effect of naratriptan and CP-122,288 on [^{35}S]GTPγS labeling (in the presence of 5-HT_{1A} and 5-$HT_{1B/1D}$ receptor blockers) is unexpected. The binding affinities (K_D) of naratriptan and CP-122,288 for 5-HT_{1F} binding sites are 4 nM (10) and 1.6 nM (14), respectively (i.e., comparable to the affinities of the 5-HT_{1A} and 5-$HT_{1B/1D}$ agonists used in this study for their respective receptors). Furthermore, 5-HT_{1D} and 5-HT_{1F} recognition sites are present at comparable densities in guinea pig brain (14). Several explanations might account for the absence of effect of naratriptan and CP-122,288. Agonist binding to 5-HT_{1F} sites might be more sensitive to high concentrations of GDP, or 5-HT_{1F} receptor might be coupled to a different subtype of $G_{i/o}$ protein than the other receptors visualized with [^{35}S]GTPγS autoradiography. Adham *et al.*

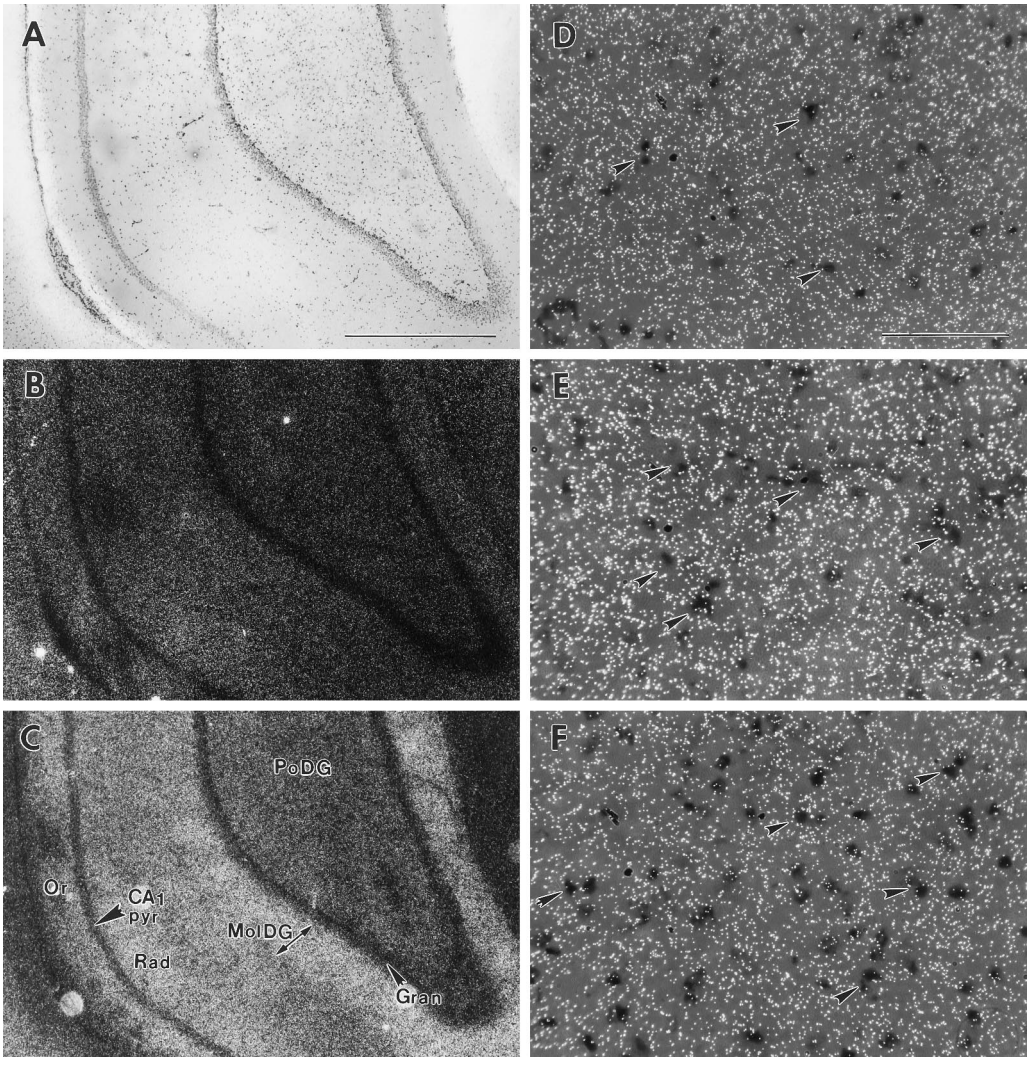

Fig. 8. High-resolution autoradiography of [^{35}S]GTPγS binding sites in guinea pig hippocampus (B and C), superficial gray layer of the superior colliculus (D), globus pallidus (E), and substantia nigra (F). [^{35}S]GTPγS-labeled slides have been coated with Kodak NTB2 liquid emulsion as described in Experimental Procedures. A, Basic fuchsin-counterstained cell nuclei in the ventral hippocampus. B, Autoradiographic silver grains (*white dots* under dark field illumination) over the same section, incubated with [^{35}S]GTPγS in the absence of agonist: labeling is low and homogeneous, except over the cell bodies, where only background labeling is observed. In the presence of 1 μM 8-OH-DPAT (C), binding is enhanced markedly in the strata oriens (*Or*) and radiatum (*Rad*) of Ammon's horn and the molecular layer of the dentate gyrus (*MolDG*) but virtually absent over the pyramidal and granular cell layers (*CA1, pyr, and Gran*). Only a small increase is seen in the polymorph layer of dentate gyrus (*PoDG*). At a higher magnification, 8-OH-DPAT-stimulated binding of [^{35}S]GTPγS in the superior colliculus (D), as well as WIN55,212–2-stimulated binding in the globus pallidus (E) and substantia nigra (F), produces homogeneously distributed silver grains in the neuropil of these areas, whereas only very few grains are observed over the cell bodies (*arrowheads*). Scale bars, 1 mm (A–C) and 0.2 mm (D–F).

(21) reported that 5-HT$_{1F}$ receptors can couple to multiple signal transduction pathways via pertussis toxin-sensitive G proteins, possibly via distinct subtypes of G proteins. It is possible that native brain 5-HT$_{1F}$ receptors do not interact with the G protein subtype leading to inhibition of adenylate cyclase and/or with a subtype prone to detectable [^{35}S]GTPγS binding.

Concerning the difference in maximal [^{35}S]GTPγS binding with 5-HT$_{1A}$ and 5-HT$_{1D}$ agonists, it is worth noting that [^{3}H]5-CT labels a comparable density of sites in hippocampus (5-HT$_{1A}$ sites) and substantia nigra (5-HT$_{1D}$ sites) (13). It is thus likely that 5-HT$_{1A}$ receptors possess a larger amplification factor than 5-HT$_{1D}$ receptors. This raises the issue of whether drugs assumed to be selective for 5-HT$_{1D}$ receptors would activate more 5-HT$_{1A}$ receptors *in vivo* than expected on the basis of their selectivity ratios. There are, however, very few systems in which this possibility can be explored because it is difficult to determine the contribution of the initial G protein activation step if distinct pathways lead from receptor stimulation to functional response. This question is nevertheless of interest because for most of the available drugs, the 5-HT$_{1A}$/5-HT$_{1D}$ selectivity ratio (indicated in parentheses) is rather low: L-694,247 (3) (Ref. 22), naratriptan (106) (Ref. 10), sumatriptan (10–60) (Refs. 10 and 23), CP-122,288 (6) (Refs. 10 and 23), and GTI (21) (Ref. 24). 5-(Nonyloxy)-tryptamine, with a 5-HT$_{1A}$/5-HT$_{1D}$ affinity ratio of 260 (Ref. 25), is one of the most selective agents, but at concentrations of >10 μM, it activates more 5-HT$_{1A}$ than 5-HT$_{1D}$ receptor-linked G proteins.

When compared with *in vitro* autoradiography with the use of radiolabeled receptor ligands, [^{35}S]GTPγS autoradiography offers significant advantages, with only a few drawbacks. The major disadvantage is the fact that the current method is not applicable to receptors coupled to pertussis toxin-insensitive G proteins or even to some receptors coupled to G_i or G_o (e.g., 5-HT$_{1F}$ receptors). Its quantification might also be less reliable because it is not known with certainty whether all subtypes of G proteins respond in the same manner in this system. The cause for the differences between rat and guinea pig brains is also unknown, and it cannot be ruled out that similar differences exist between brain regions and were unnoticed in this and previous reports. Minor shortcomings are the smaller resolution of ^{35}S versus ^{3}H (used to label most receptor ligands) and the fact that film darkening with ^{35}S depends on the section thickness (a very reproducible cryostat-microtome should thus be used; only the first 4–5 μm of a ^{3}H-labeled section are responsible for film exposure). The main advantage of [^{35}S]GTPγS autoradiography is that one

can determine agonist efficacies in various brain regions. This technique can be used even in the absence of a suitable receptor radioligand and requires short exposure times (1–2 days versus 2 weeks to 6 months for conventional autoradiography). The current report also shows that ^{35}S-GTPγS-labeled sections can be directly coated with nuclear emulsion and potentially resolve receptor distribution at the cellular level. This can be achieved only because [^{35}S]GTPγS binds to its target in a virtually irreversible manner (26). In contrast, receptor radioligand binding is usually reversible (even more so at the temperature required to coat the slides with nuclear emulsion), and cross-linking to the receptor can be performed only for selected ligands (in general peptides). The light microscopic distribution of [^{35}S]GTPγS binding stimulated by 8-OH-DPAT is very similar to that reported previously for [^{3}H]8-OH-DPAT binding sites (27). Interestingly, most of the [^{35}S]GTPγS binding to activated G proteins occurs in the neuropil (in the superior colliculus, globus pallidus, and substantia nigra) or on the cell processes (hippocampus), which is in agreement with the expected distribution of G proteins (28, 29). Finally, [^{35}S]GTPγS autoradiography, coupled with selective antibodies or peptides (30), can potentially be used to investigate which G protein subtypes are coupled to different receptors in the brain and regional differences in this coupling.

Acknowledgments

We thank Dr. Ivana Delalle for her help in the light-microscope experiments.

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