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Differential Inactivation and G Protein Reconstitution of Subtypes of [^{3}H]5-Hydroxytryptamine Binding Sites in Brain

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SUMMARY

The sulfhydryl reagents *p*-chloromercuribenzoate and *N*-ethylmaleimide (NEM) inactivate high affinity [^{3}H]serotonin ([^{3}H]5-HT) binding to bovine and rat brain membranes in a concentration-dependent manner. In both species, 15–25% of total specific high affinity [^{3}H]5-HT binding is relatively insensitive to NEM. This study examines the NEM sensitivity of the various high affinity [^{3}H]5-HT binding subtypes, using selective ligands, tissues, and pharmacological masks to study each subtype. Reconstitution of NEM-inactivated binding by addition of GTP-binding proteins (G proteins, G_i and G_o) is also described. Agonist binding to 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} sites in rat brain and to 5-HT_{1A} and 5-HT_{1D} sites in bovine brain is sensitive to NEM. Binding of [^{3}H]dihydroergotamine and [^{125}I]iodocyanopindolol, both of which are weak partial agonists, to 5-HT_{1B} sites is relatively insensitive to NEM. Binding of [^{3}H]5-HT to 5-HT_{1C} sites in rat and bovine brain and choroid plexus is relatively insensitive to NEM. In the presence of spiperone to mask binding of 5-HT_2 sites, binding of antagonist ([^{3}H]mesulergine) to 5-HT_{1C} sites is also insensitive to NEM. Likewise, binding of the agonist [^{3}H]4-bromo-2,5-dimethoxyphenylisopropylamine and of the antagonist [^{3}H]ketanserin to 5-HT_2 sites is not inhibited by NEM treatment of membranes. These findings suggest that agonist binding to 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} sites is sensitive to NEM alkylation. Binding of neither agonist nor antagonist to 5-HT_{1C} and 5-HT_2 sites is sensitive to NEM. Inability of high concentrations of a variety of ligands to protect the sensitive binding sites against NEM inactivation indicates that the critical sulfhydryl group(s) are not located in the ligand binding domain of the NEM-sensitive binding sites. When membranes are treated with NEM, displacement of [^{125}I]iodocyanopindolol by 5-HT is no longer sensitive to 5′-guanylyl imidodiphosphate (Gpp(NH)p). Gpp(NH)p sensitivity of agonist displacement of 5-HT_{1B} binding to NEM-treated membranes is restored by addition of purified guanine nucleotide binding proteins (G_i plus G_o). In addition, NEM-inactivated binding to 5-HT_{1A} and 5-HT_{1D} sites can be restored by addition of G_i plus G_o. These data suggest that NEM exerts its effects on 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} binding sites by inactivating the G protein(s) associated with the 5-HT receptor subtypes.

Involvement of sulfhydryl groups in binding of neurotransmitters to receptors has been reported in a number of systems, including β-adrenergic (1, 2), dopaminergic (3–5), muscarinic (6, 7), GABA-ergic (8), α_2-adrenergic (9), and adenosine receptor systems (10, 11). The dopamine D_1 receptor appears to have a sulfhydryl group at the ligand binding site (5, 12), whereas the sulfhydryl groups that influence binding to GABA-B, α_2- and β-adrenergic receptors are distinct from the ligand binding site (2, 8, 9). Binding to NEM-inactivated α_2-adrenergic and GABA-B binding sites has been restored by addition of purified G proteins (G_i and G_o) to the brain preparations (8, 9).

Central nervous system serotonin (5-HT) binding sites have been divided into 5-HT_1 and 5-HT_2 sites on the basis of their relative affinities for [^{3}H]5-HT and [^{3}H]spiperone (13). In rat brain, high affinity [^{3}H]5-HT binding sites have been further subdivided into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, and 5-HT_{1D} sites (see Ref. 14 for review). Of these subtypes, the 5-HT_{1B} site appears to be present only in rodents (15, 16).

Although several groups have reported NEM sensitivity of [^{3}H]5-HT binding in brain, neither the sulfhydryl specificity nor the mechanism of this effect has been fully characterized (17–19). More recently, Hall *et al.* (20) showed that binding of [^{3}H]5-HT and [^{3}H]DPAT in rat brain are differentially sensitive to NEM in different brain regions, but no further subtype analysis was described. The presence or absence of critical sulfhydryl groups appears to be a useful way to examine molecular similarities and differences among [^{3}H]5-HT binding sites. Accordingly, we examined the effects of NEM and PCMB, two

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DE, dihydroergotamine; DPAT, 8-hydroxy-2-(di-propylamino)tetralin; DOB, 4-bromo-2,5-dimethoxyphenylisopropylamine; 5-HT, 5-hydroxytryptamine (serotonin); G proteins, guanine nucleotide-binding proteins; G_i and G_o, G proteins from bovine brain characterized by α subunits of 41 and 39 kDa, respectively; Gpp(NH)p, 5′-guanylyl imidodiphosphate; ICYP, iodocyanopindolol; NEM, *N*-ethylmaleimide; PCMB, *p*-chloro-mercuribenzoate; RU 24969, 5-methoxy3(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole succinate; (±)-21-009, 4-[3-terbutyl-amino-2-hydroxypropoxyl]-indole-2-carbonic-acid-isopropylester; CYP, cyanopindolol; PI, phosphatidylinositol; GTPγS, guanosine 5′[γ-thio] triphosphate; DTT, dithiothreitol.

reagents that react with sulfhydryl groups via different mechanisms, on high affinity [^{3}H]5-HT binding subtypes in membranes from rat and bovine brain.

We report here that binding to rat and bovine 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} sites is sensitive to sulfhydryl modification, whereas 5-HT_{1C} and 5-HT_2 binding sites are insensitive to NEM alkylation. We provide evidence that NEM sensitivity is conferred by association of these binding sites with G proteins, inasmuch as NEM-inactivated agonist binding can be restored by addition of purified G proteins to the membrane preparation.

Materials and Methods

Bovine brains were obtained from a local slaughterhouse. Rat brains were obtained from PelFreez (Rogers, AK). Tris, 5′-guanylyl imidodiphosphate sodium (GppNHp) and N-ethylmaleimide were from Sigma. PCMB was from CalBiochem (La Jolla, CA). Serotonin creatinine sulfate (5-HT) was from Regis Chemical Co. (Morton Grove, IL). [^{3}H]5-HT (25–30 Ci/mmol), [^{3}H]ketanserin (72.3 Ci/mmol), [^{3}H]DPAT (157 Ci/mmol), [^{3}H]DOB (22.3 Ci/mmol), and [^{125}I]ICYP (>2000 Ci/mmol) were from New England Nuclear (Boston, MA). [^{3}H]Mesulergine (85 Ci/mmol) and [^{35}S]GTPγS (10–30 Ci/mmol) was from Amersham (Arlington Heights, IL). [^{3}H]DE was custom synthesized by CEA (Saclay, France) and purified by C-18 reverse phase high performance liquid chromatography (21). Unlabeled DPAT was purchased from Research Biochemicals Inc. (Wayland, MA). Phentolamine was the gift of CIBA-Geigy (Summit, NJ).(±)21-009 was the gift of Sandoz (Basel, Switzerland). RU 24969 was the gift of Roussel-Uclaf (Paris, France). Spiroxatrine was the gift of Janssen (Beerse, Belgium). Glass fiber filters (GF/C) were from Whatman (Clifton, NJ).

Tris buffers were calibrated at room temperature (25°). TEM buffer consists of 150 mM Tris·HCl, pH 7.4, 1 mM EDTA, and 5 mM $MgSO_4$.

Preparation of Tissues

Membrane fractions were prepared from bovine frontal cortex by the method described by VandenBerg *et al.* (22). Cow brains were obtained from a local slaughterhouse and transported to the laboratory immersed in ice-cold saline. We defined bovine frontal cortex as that portion of the cortex rostral to the caudate. Underlying white matter was carefully dissected away in the coldroom and discarded. The cortical material was immediately frozen in small portions in plastic bags on dry ice and stored at −80°. For preparation of membrane fractions from bovine cortical material, the tissue was thawed in 10 volumes (ml/g) of ice-cold 0.32 M sucrose/2.5 mM Tris, pH 6.95, then homogenized (15–20 strokes) in a Dounce homogenizer. The homogenate was centrifuged (1000 × g_{max}) in a Sorvall SA600 rotor. The supernatant was then centrifuged at 60,000 × g_{max} for 25 min in a Beckman 50.2Ti rotor. The pellets were resuspended in ice-cold water (25–30 ml) then centrifuged at 60,000 × g_{max}. Each pellet was resuspended in 3 ml of 0.32 M sucrose, 2.5 mM Tris, pH 6.95, and incubated at 30° for 20 min. Ice-cold water (22–25 ml) was added to each suspension, and the mixture was centrifuged again. Resulting pellets were resuspended and combined in ice-cold water, the final volume equaling 2.38 times the original wet weight of tissue. This suspension was sonicated briefly (5 sec) then aliquoted, frozen on dry ice, and stored at −80°. Protein concentration of the resulting membrane preparation was typically 8–12 mg/ml.

Cortices and hippocampi from frozen rat brains were removed and suspended in 40 volumes of ice-cold 50 mM Tris, pH 7.4. The suspension was subjected to Polytron homogenization (setting 7 for 15 sec), then centrifuged for 20 min at 40,000 × g_{max} (Sorvall SA600 rotor). The resulting pellets were resuspended in 10 volumes of Tris buffer by Polytron homogenization, as above, then incubated at 37° for 10 min. Thirty volumes of ice-cold Tris buffer were then added, and the samples were centrifuged as above. Resulting pellets were resuspended by Polytron homogenization in 2× concentrated TEM buffer to a final volume equalling 5 times the original wet weight (200 mg of wet weight/ml suspension). The suspension was aliquoted, frozen on dry ice, and stored at −80° until used.

Rat choroid plexes were removed and stored at −80° before preparation of a membrane preparation. The tissue (20 mg) was sonicated (probe sonicator) in 2 ml of ice-cold 0.32 M sucrose, then centrifuged at 750 × g for 15 min. Supernatants were centrifuged again at 750 × g for 15 min. This supernatant was then centrifuged at 35,000 × g for 20 min. All pellets were resuspended in 2 ml of 50 mM Tris, pH 7.4, then incubated at 37° for 15 min. The mixture was centrifuged at 49,000 × g for 20 min. The pellet was resuspended in 2 ml of 50 mM Tris, pH 7.4, before use in assays.

Membrane Treatment with Sulfhydryl Reagent

Membranes were preincubated with varying concentrations of NEM or PCMB in a total volume of 0.3 ml in TEM buffer containing pargyline (10 μM) with varying concentrations of NEM at 30° for 20 min. Preliminary studies showed that the maximal effect of either reagent was complete within this time period (data not shown). Radioactive ligands and competing drugs were then added for radioligand binding as described below.

Radioligand Binding Assays

All radioligand binding assays were performed in TEM buffer containing 10 μM pargyline and 0.1 mM ascorbic acid. Previous studies (23) demonstrated that this concentration of ascorbate fully protected ligands against oxidation without interfering with binding. Final incubation volume was 0.5 ml, unless otherwise indicated, containing 0.1–0.2 mg of bovine or rat brain membrane preparation or 0.03–0.05 mg of choroid plexus membrane preparation.

[^{3}H]5-HT binding. [^{3}H]5-HT was added to a final concentration of 2 nM and incubated at 30° for 20 min before filtration (Brandell Cell Harvester) over glass fiber filters (GF/C) and determination of filter-bound radioactivity. In order to restrict binding of [^{3}H]5-HT to a particular subtype, unlabeled site-specific ligands ("masking agents") were included in the assay. Binding of [^{3}H]5-HT to 5-HT_{1B} and 5-HT_{1D} sites was examined by inclusion of 100 nM DPAT or 100 nM spiroxatrine to block radioligand binding to 5-HT_{1A} sites and 100 nM mesulergine to block binding to 5-HT_{1C} sites. [^{3}H]5-HT binding to 5-HT_{1C} sites was carried out in the presence of 100 nM DPAT and 300 nM RU 24969 to mask binding to 5-HT_{1A} and 5-HT_{1B} plus 5-HT_{1D} sites, respectively. Experiments in our laboratory have shown that under these conditions, although specific binding is low, binding displacement of [^{3}H]5-HT by various agents is consistent with the drug displacement profiles generated at other 5-HT_{1C} sites. In order to restrict binding of [^{3}H]5-HT to 5-HT_{1D} sites in rat brain preparations, (±)-21-009 (100 nM) was added in addition to DPAT and mesulergine. In all cases, nonspecific binding was defined by inclusion of 10 μM 5-HT in parallel assay tubes.

[^{3}H]DPAT binding. Binding of [^{3}H]DPAT (0.5–0.7 nM) was carried out in a final volume of 0.5 ml at 30° for 20 min. Nonspecific binding was estimated by inclusion of 10 μM unlabeled 5-HT in the assay.

[^{3}H]DE binding. [^{3}H]DE was used to label 5-HT_{1B} sites, as described by Hamblin *et al.* (21). [^{3}H]DE was added to a final concentration of 80–100 pM, unless otherwise indicated, in a final volume of 1 ml. Spiroxatrine (100 nM) and phentolamine (100 nM) were added to mask binding to 5-HT_{1A} and α-adrenergic sites, respectively. Nonspecific binding was determined by inclusion of (±)-21-009 (100 nM) in replicate assay tubes. Incubation was carried out at 37° for 45 min before filtration over GF/C filters that were pretreated with 0.1% bovine serum albumin.

[^{125}I]ICYP binding. [^{125}I]ICYP was used to label 5-HT_{1B} sites (24). Sensitivity of binding of 90 pM [^{125}I]ICYP binding to NEM was determined in the presence of 3–30 μM isoproterenol, to mask binding to β-adrenergic sites as reported by Hoyer *et al.* (24) and confirmed in this laboratory in preliminary experiments. Incubation was at 25° for 30 min. These incubation conditions differ from those originally described (24) and are based on recent studies in our laboratory examining

association and dissociation kinetics of binding of [^{125}I]ICYP to 5-HT$_{1B}$ sites.[1]

[^{3}H]Mesulergine binding. Binding of [^{3}H]mesulergine to 5-HT$_{1C}$ sites was carried out at a final concentration of 65–90 pM. Spiperone (30 nM) was included in the assay to mask binding to 5-HT$_2$ sites. Incubation was at 30° for 15 min. Under these conditions, [^{3}H]mesulergine labels a population sites with a B_{max} of approximately 25 fmol/mg of protein in both species. This binding is characteristic of 5-HT$_{1C}$ binding. Parameters from binding experiments carried out in bovine cortex are as follows: [^{3}H]mesulergine, $K_{d(app)}$ = 1.5 nM; 5-HT, K_i = 7 nM; spiperone, K_i = 350–700 nM; *d*-lysergic acid diethylamide, K_i = 5 nM; and ketanserin, K_i = 30 nM. Similar results are obtained in rat cortex (data not shown).

[^{3}H]Ketanserin binding. Binding of [^{3}H]ketanserin to 5-HT$_2$ sites was carried out at a concentration of 0.5–0.6 nM, using 10 μM 5-HT to define nonspecific binding. Incubation was at 30° for 15 min.

[^{3}H]DOB binding. [^{3}H]DOB binding to 5-HT$_2$ sites (25) was carried out at a concentration of 0.4 nM. Incubation was at 37° for 15 min. Specific binding was defined by inclusion of 1 μM cinanserin and was determined to be 52% of total binding.

Purification of G Proteins

G proteins were purified from bovine brain by modifications of the methods of Sternweis and Robishaw (26) and Neer *et al.* (27), as described by Kim and Neubig (28). Fractions were assayed for binding of [^{35}S]GTPγS (26). Fractions from the final heptylamine-Sepharose column were pooled on the basis of composition judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation of proteins. In the experiments shown here, $G_o\alpha$ and $G_i\alpha$ were present in a ratio of approximately 10:1 (G_o:G_i). Total G protein comprised 60–80% of the protein bands visualized by Coomassie blue staining.

Reconstitution of G Proteins with Treated Membranes

Membranes prepared as described above were thawed and diluted 4-fold with TEM buffer, and water or NEM (1 mM, final concentration) was added. The mixtures were incubated for 15 min at 30°, then centrifuged at 100,000 × *g* for 10 min in a Beckman TL-100.3 rotor. Pellets were resuspended in ice-cold TEM buffer and DTT was added to a final concentration of 1.5 mM before centrifugation, as above. The pellets were resuspended and centrifuged a final time in TEM buffer then resuspended to the original pre-dilution volume with 2× concentrated TEM. The protein concentration in this suspension was typically 12–15 mg/ml. G protein was added as the ratio 0.06 mg of G protein/1.4–1.8 mg of membrane protein (to give a theoretical ratio of approximately 1000:1, G protein: receptor, described by Kim and Neubig (28) as optimal for reconstitution). The mixtures were then sequentially incubated on ice for 10 min, sonicated in a bath sonicator for 5 sec, and incubated at 30° for 5 min, then on ice for 30–45 min before use in the binding assay. The G_i plus G_o fraction contained cholate, contributing an approximate final concentration of 0.005% cholate to the binding assay. This amount of detergent did not affect control binding. Therefore, cholate or buffer alone was added as control for G protein addition.

Protein Determination

Protein concentrations were estimated using the method of Bradford (29) with immunoglobulin G as standard.

Data Analysis

Nonlinear regression analysis was performed using the program LIGAND (30) adapted for an IBM PC (Elsevier BIOSOFT).

Results

Addition of NEM or PCMB to bovine or rat brain cortical membranes results in a reduction of [^{3}H]5-HT binding to high affinity [^{3}H]5-HT binding sites. Fig. 1 shows the results of experiments in which membranes were preincubated with various concentrations of NEM or PCMB at 30° for 20 min before addition of 2 nM [^{3}H]5-HT. PCMB inactivates all binding of [^{3}H]5-HT to bovine and rat membranes with EC_{50} values (concentration producing 50% inhibition of binding) of 25 μM in each case. In contrast, in both species, NEM inactivates only about 70 to 85% of the [^{3}H]5-HT binding, despite extensive preincubation of membranes with the sulfhydryl reagent. Although, in the experiments shown, NEM was present during the binding assay, similar results were obtained when membranes were washed before binding (data not shown), suggesting that the effect of NEM on binding is indeed via covalent modification of membrane components. At a very high concentration (50 mM), NEM inactivates 100% of high affinity binding (cf. Fig. 2). However, at such high concentrations, NEM reacts with other functional groups present on proteins such as amine groups and can no longer be considered a thiol-specific reagent (31). The presence of high affinity [^{3}H]5-HT binding that occurs in the presence of 1 mM NEM suggests that some portion of high affinity [^{3}H]5-HT$_1$ receptor binding may be resistant to NEM alkylation.

In order to determine whether the effects of NEM and PCMB were attributable to their effects on sulfhydryl groups, 1 mM DTT was added to the membranes before addition of the sulfhydryl reagents (Fig. 1). Under these conditions, the effect of NEM is prevented by DTT, indicating that NEM inactivates receptors via thiol alkylation. In contrast, the effects of PCMB are not entirely blocked by prior addition of DTT to the assay. This is more pronounced in the bovine preparation, where substoichiometric amounts of PCMB affect binding in the presence of 1 mM DTT. These experiments suggested that the effects of NEM on [^{3}H]5-HT binding are probably mediated via sulfhydryl alkylation, whereas PCMB has both sulfhydryl and non-sulfhydryl-mediated effects on binding. For these reasons, we used NEM to further characterize the sensitive sulfhydryl group(s) associated with high affinity [^{3}H]5-HT binding sites.

Subtype analysis: effects of NEM on 5-HT$_{1A}$ binding sites. Fig. 2 shows the effects of increasing concentrations of NEM on the various 5-HT$_1$ subtypes present in bovine and rat brain. As described above, and shown here for comparison, binding of [^{3}H]5-HT to both rat and bovine membranes exhibits a biphasic sensitivity to treatment with NEM (Fig. 2, A and B, *solid squares*). Binding of [^{3}H]DPAT, a selective 5-HT$_{1A}$ ligand, is highly sensitive to NEM treatment of membranes. The EC_{50} values of NEM for inactivation of 5-HT$_{1A}$ sites in bovine and rat brain are 39 and 33 μM, respectively.

Effects of NEM on 5-HT$_{1B}$ and 5-HT$_{1D}$ binding sites. When unlabeled DPAT and mesulergine are included to mask binding to 5-HT$_{1A}$ and 5-HT$_{1C}$ sites, respectively, [^{3}H]5-HT binds to one (bovine) or two (rat) additional sites. Although at the inception of this study it was thought that the only site labeled in rat cortex under these conditions was the 5-HT$_{1B}$ site, we have recently found that only approximately 60% of [^{3}H]5-HT specific binding in the presence of mesulergine and DPAT in rat cortex has high apparent affinity for (±)-21-009 (K_i = 0.3 nM), RU 24969 (K_i = 0.38 nM) and (±)-CYP (K_i = 0.36 nM). This displacement profile is characteristic of the 5-HT$_{1B}$ binding sites. The remaining 40% of binding is insensitive to (±)21-009. After more detailed analysis, it has become ap-

[1] G. L. Tan and R. D. Ciaranello, unpublished observations.

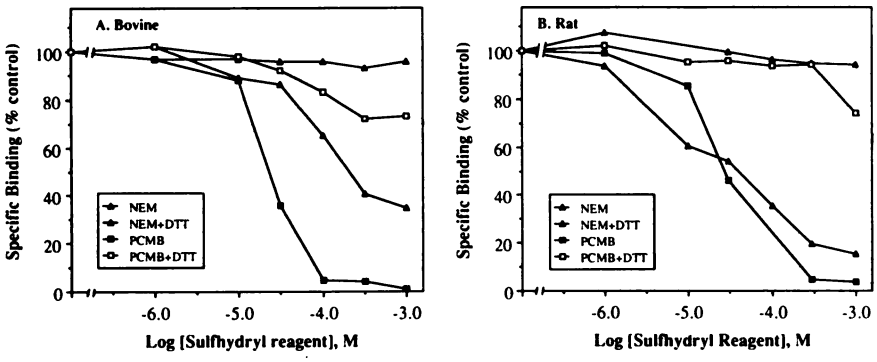

Fig. 1. Effect of DTT preincubation on NEM or PCMB inactivation of [^{3}H]5-HT binding to bovine cortical and rat cortical plus hippocampal membranes. Bovine (A) or rat (B) cortical membranes were mixed in the absence (*closed symbols*) or presence (*open symbols*) of DTT (1mM final concentration) in test tubes in an ice bath. Varying concentrations of NEM (*triangles*) or PCMB (*squares*) were added, and the samples were incubated for 20 min at 30° before addition of [^{3}H]5-HT and, to determine nonspecific binding, unlabeled 10 μM 5-HT. Binding was carried out at 30° for 20 min, before filtration, as described in Materials and Methods. *Points* are means of triplicate samples. Control values (100%): bovine NEM, 94 fmol/mg of protein; bovine PCMB, 88 fmol/mg of protein; rat NEM, 71 fmol/mg of protein; and rat PCMB, 81 fmol/mg of protein.

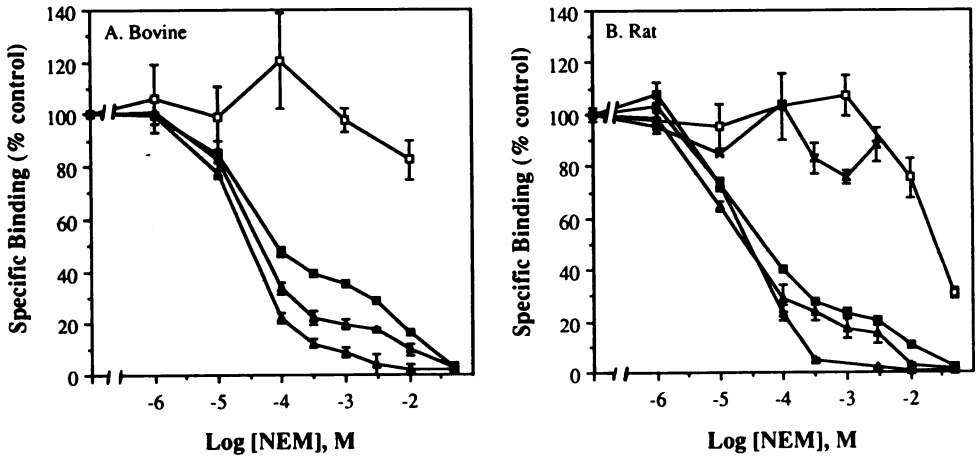

Fig. 2. NEM sensitivity of 5-HT$_1$ binding site subtypes in bovine and rat brain. Membranes (0.25–0.4 mg of protein) were incubated with various concentrations of NEM for 20 min at 30° before addition of radiolabeled ligand and masking agents as indicated and described in Materials and Methods. A, Bovine frontal cortex: ■, Binding of [^{3}H]5-HT (2–2.4 nM) represents binding to all 5-HT$_1$ subtypes; △, binding of [^{3}H]DPAT (0.7 nM) represents binding to 5-HT$_{1A}$ subtypes; □, binding of [^{3}H]mesulergine (0.8 nM) in the presence of unlabeled spiperone represents binding to 5-HT$_{1C}$ sites; ▲, binding of [^{3}H]5-HT in the presence of unlabeled DPAT and mesulergine represents binding to 5-HT$_{1D}$ sites. B, Rat cortex plus hippocampus. *Symbols* are identical to those described for bovine frontal cortex, except that ▲ represents binding to a combination of 5-HT$_{1B}$ and 5-HT$_{1D}$ sites, and ×, binding of [^{3}H]5-HT in the presence of unlabeled DPAT and RU 24969 represents binding to 5-HT$_{1C}$ subtype. Data *points* and *bars* represent mean ± standard error, based on two or three separate experiments carried out in triplicate and expressed as per cent control specific binding.

parent that this represents binding to 5-HT$_{1D}$ sites[2] (32). Fig. 2 shows that the mixture of 5-HT$_{1B}$ and 5-HT$_{1D}$ sites present in the rat preparation is sensitive to NEM (EC_{50} = 20 μM; Fig. 2B). When binding to 5-HT$_{1B}$ sites in rat membranes is masked by the presence of 100 nM (±)-21-009 in addition to DPAT and mesulergine, [^{3}H]5-HT now labels only 5-HT$_{1D}$ sites (K_i values: RU 24969, 140 nM; yohimbine, 100 nM; CYP, >10,000 nM). These sites are also sensitive to NEM (EC_{50} = 67 μM; data not shown). In bovine brain, 5-HT$_{1D}$ binding sites are similarly sensitive to NEM (EC_{50} = 56 μM; Fig. 2). Taken together, these data suggest that rat and bovine 5-HT$_{1D}$ sites exhibit similar sensitivity to NEM and that 5-HT$_{1B}$ sites may be slightly more sensitive to NEM alkylation.

The 5-HT$_{1B}$ site is the only NEM-sensitive [^{3}H]5-HT binding site for which both a high affinity radiolabeled agonist and antagonist are readily available. As previously described, binding of 2 nM [^{3}H]5-HT to rat membranes in the presence of DPAT and mesulergine masks is directed to a mixture of 5-HT$_{1B}$ and 5-HT$_{1D}$ sites, with 5-HT$_{1B}$ sites accounting for approximately 60% of the binding. 5-HT$_{1B}$ sites can also be labeled by [^{125}I]ICYP (24) or [^{3}H]DE (21). Although originally described as an antagonist ligand, the binding of [^{125}I]ICYP, like that of [^{3}H]DE, has some characteristics suggesting that it is a weak partial agonist.[3] We used these observations to compare NEM effects on agonist and antagonist binding of 5-HT$_{1B}$ sites. In contrast to agonist binding to the combination of 5-HT$_{1B}$ and 5-HT$_{1D}$ sites (Fig. 2), 5-HT$_{1B}$ sites labeled with [^{3}H]DE or [^{125}I]ICYP are relatively insensitive to NEM (Fig. 3). These results indicate a differential effect of NEM on agonist and partial agonist or antagonist binding 5-HT$_{1B}$ sites.

Effects of NEM on 5-HT$_{1C}$ binding sites. The third high affinity [^{3}H]5-HT binding site identified in rat and bovine brain, the 5-HT$_{1C}$ site, has a high affinity for mesulergine (33). Mesulergine also binds to 5-HT$_2$ sites (34), but this binding can be masked by inclusion of unlabeled spiperone in the assay.

[2] P. I. Adriaenssens, M. W. Hamblin, and R. D. Ciaranello, unpublished observations.

[3] K. Ariani, M. W. Hamblin, and R. D. Ciaranello, manuscript in preparation.

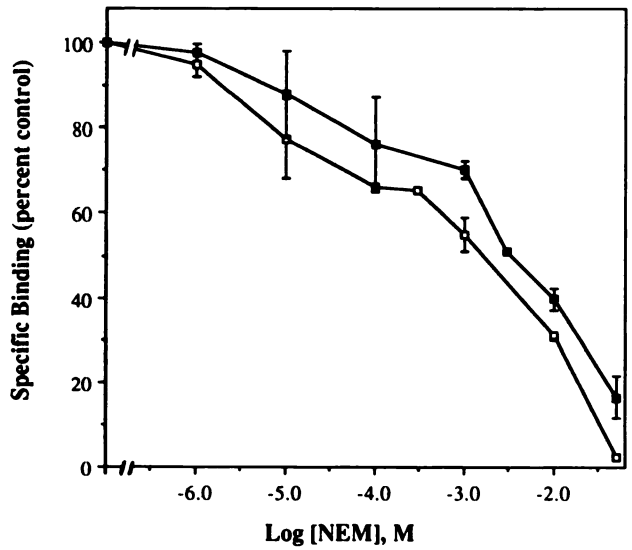

Fig. 3. Sensitivity of rat 5-HT$_{1B}$ binding sites to NEM. Membranes were preincubated with various concentrations of NEM and assayed for binding of [^{3}H]DE in the presence of unlabeled spiroxatrine and phentolamine (□), and [^{125}I]ICYP in the presence of unlabeled isoproterenol (■), as described in Materials and Methods. Control specific binding: [^{3}H]DE, 36 fmol/mg of protein; [^{125}I]ICYP, 29 fmol/mg of protein.

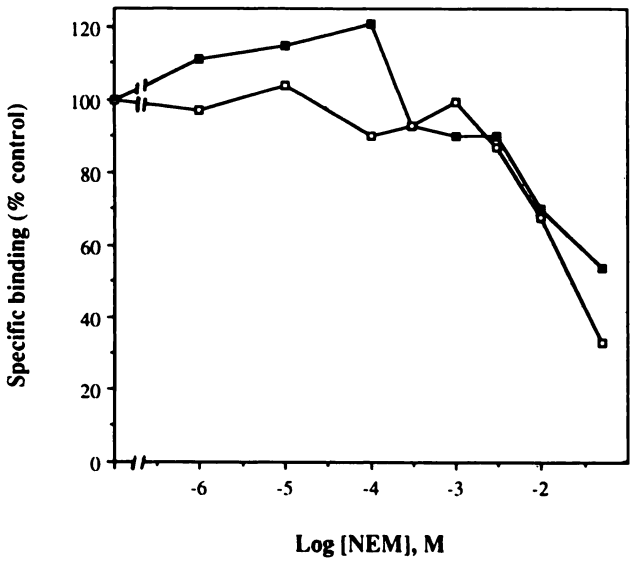

Fig. 4. Effect of NEM on binding of 5-HT$_{1C}$ binding sites in rat choroid plexus. Choroid plexus tissue (0.03 mg of protein) was incubated with various concentrations of NEM, followed by incubation with 2 nM [^{3}H]5-HT (■) or [^{3}H]mesulergine (□) in the presence of unlabeled spiperone in a total volume of 1 ml, as described in Materials and Methods.

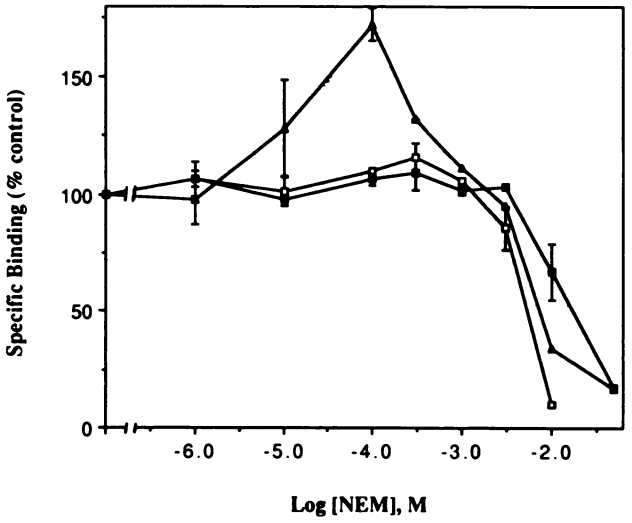

Fig. 5. Effect of NEM on binding to 5-HT$_2$ binding sites in bovine and rat brain. Membranes were incubated with NEM, as indicated, followed by incubation with 50 pM [^{3}H]ketanserin (□, bovine; ■, rat) or 0.4 nM [^{3}H] DOB (▲, rat), described in Materials and Methods. Data represent means ± standard error from two or three separate experiments.

[^{3}H]Mesulergine binding to both bovine and rat brain is insensitive to NEM (Fig. 2). To examine whether this result is due to the use of an antagonist to label 5-HT$_{1C}$ sites, we examined sensitivity of agonist binding to these sites. First, we examined NEM sensitivity of [^{3}H]5-HT binding in rat brain in the presence of DPAT, to block 5-HT$_{1A}$ binding, and RU 24969, which blocks both 5-HT$_{1B}$ and 5-HT$_{1D}$ sites. The remaining [^{3}H]5-HT binding is most likely to 5-HT$_{1C}$ sites, as shown by high affinity for mesulergine (K_i = 12 nM).[2] Fig. 2B shows that, in rat brain, the 5-HT$_{1C}$ binding measured in this manner is insensitive to NEM treatment. However, under these conditions, specific binding of [^{3}H]5-HT is very low. Therefore, we also examined NEM sensitivity of [^{3}H]5-HT binding in rat choroid plexus, a tissue in which high affinity [^{3}H]5-HT binding sites are exclusively of the 5-HT$_{1C}$ subtype (35). Fig. 4 demonstrates that, in the rat choroid plexus, binding of both [^{3}H]5-HT and [^{3}H]mesulergine are relatively insensitive to NEM. These results indicate that 5-HT$_{1C}$ binding is insensitive to NEM because of differences intrinsic to this binding site and not because of the radioligands used to label it.

Effects of NEM on 5-HT$_2$ binding sites. [^{3}H]Ketanserin is a high affinity antagonist at 5-HT$_2$ sites (36). NEM sensitivity of binding to this site was also examined. Fig. 5 shows that binding of 5-HT$_2$ sites in rat and bovine brain is relatively insensitive to NEM. Recently, [^{3}H]DOB has been described as a high affinity agonist at 5-HT$_2$ sites (25). Binding of [^{3}H]DOB was not inhibited by NEM and was even stimulated to some degree by pretreatment of membranes with NEM (Fig. 5). Whether this effect is due to an enhancement of agonist binding due to NEM-induced conformational changes at the binding site or due to a "locking" of the agonist into the binding site, as has been observed with the β-adrenergic receptor (37), is not addressed by the present study. It is clear that binding of antagonist and putative agonist to 5-HT$_2$ sites is not inactivated by NEM treatment.

Protection by ligands against NEM inactivation of high affinity [^{3}H]5-HT binding. In order to determine whether the NEM-sensitive sulfhydryl group(s) is located within the ligand binding site, we carried out experiments to determine whether occupation of the binding site by ligand could block NEM inactivation of subsequent ligand binding. Membranes were incubated with an excess of various unlabeled ligands, then treated with NEM, before extensive washing and subsequent binding of [^{3}H]5-HT. Fig. 6 shows that preincubations with the ligands 5-HT, DPAT, (±)-21-009, RU 24969, mesulergine, or spiperone were no more effective than buffer in protection against NEM inactivation of high affinity [^{3}H]5-HT binding sites. After treatment with some of the ligands, binding of [^{3}H]5-HT was reduced relative to samples not treated with either NEM or protecting ligand (see legend to Fig. 6), presumably due to retention of some of the protective drugs despite seven sequential washings of the membranes.

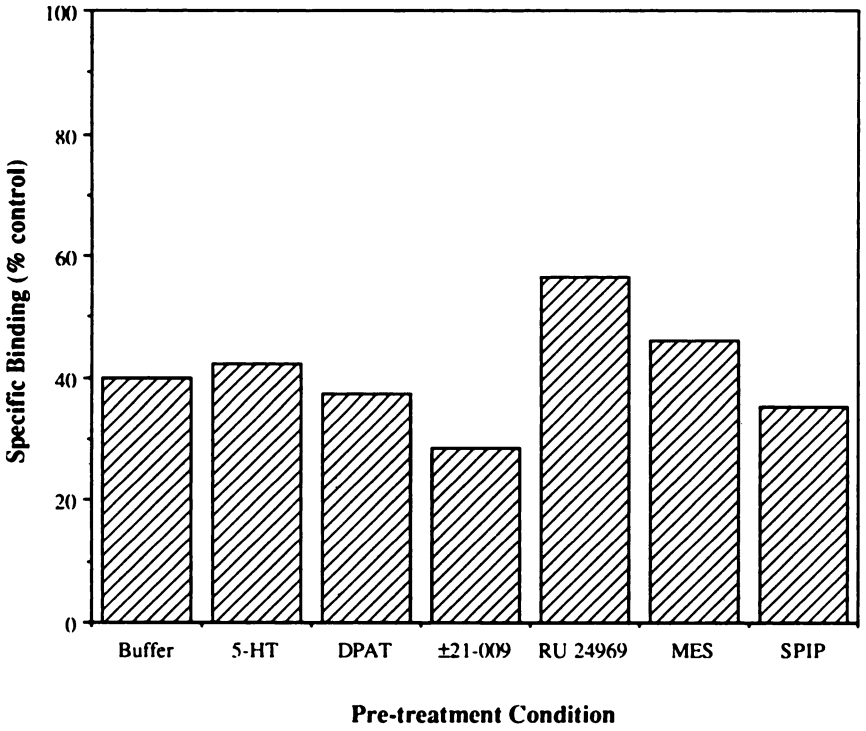

Fig. 6. Effect of pre- and coincubation with ligands on NEM binding site inactivation. Rat cortical plus hippocampal membranes (4 mg of protein in 0.2 ml) were diluted with 1 ml of 150 mM Tris, pH 7.4, and centrifuged (13,000 × *g*, 5 min), and the pellets were resuspended to 0.2 ml in the same buffer. Tris buffer or the ligand indicated (100 μM, final concentration) was added, followed by incubation at 30° for 20 min. Tris buffer (control) or NEM (1 mM, final concentration) was then added, as indicated, and the tubes were again incubated at 30° for 20 min. Ice-cold 150 mM Tris, pH 7.4, containing 0.1 mM ascorbate (1 ml) was added to each tube, followed by centrifugation (13,000 × *g*, 5 min). This washing step was repeated a total of seven times. Pellets were then resuspended in TEM buffer and assayed for [^{3}H]5-HT binding (1.9 nM) as described in Materials and Methods. Data are pooled from two separate experiments, each carried out in triplicate, and are presented as percentage of control tubes that were preincubated with the same ligand, followed by buffer. Control values after preincubation with the following were: buffer, 124 fmol/mg of protein; 5-HT, 122 fmol/mg of protein; DPAT, 107 fmol/mg of protein; (±)-21-009, 85 fmol/mg of protein; RU 24969, 52 fmol/mg of protein; mesulergine (*MES*), 90 fmol/mg of protein; spiperone (*SPIP*), 90 fmol/mg of protein.

Nonetheless, the proportion of NEM-sensitive [^{3}H]5-HT binding sites was approximately the same as under control conditions. In addition, neither unlabeled DPAT nor spiperone was effective in preventing inactivation of [^{3}H]DPAT binding by NEM. Taken together, these results indicate that the critical sulfhydryl group(s) is probably not located within the [^{3}H]5-HT binding domain. They do not, however, address the question of whether thiol groups outside the ligand binding site exist on the receptor molecule.

Effect of NEM on Gpp(NH)p sensitivity of binding to 5-HT$_{1B}$ sites. In other receptor binding systems having critical sulfhydryl groups located distant to the ligand binding site, the pattern has emerged that sulfhydryl sensitivity is conferred by association of the receptor with G proteins (8, 9). Although early studies of guanine nucleotide sensitivity of 5-HT$_1$ binding were equivocal, as the various receptor subtypes have been better delineated, evidence for G protein linkage of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor subtypes has become clearer. In order to study the possible involvement of G protein(s) in the NEM sensitivity of high affinity [^{3}H]5-HT binding, we carried out the experiments shown in Fig. 7. The curve representing competition of the 5-HT$_{1B}$ antagonist [^{125}I]ICYP binding to rat brain membranes by unlabeled 5-HT is adequately fit by nonlinear regression analysis by assuming the presence of a single binding site. The curve is shifted to the right by addition of the nonhydrolyzable GTP analog Gpp(NH)p to the assay mix, consistent with earlier observations of G protein involvement with 5-HT$_{1B}$ binding sites (21). Treatment of membranes with NEM results in a similar shift to the right of the competition curve. When Gpp(NH)p is added to the assay in addition to NEM, there is no further right shift of 5-HT competition of [^{125}I]ICYP binding. Similar results were obtained using [^{3}H]DE as ligand (data not shown). Although these data do not constitute proof of G protein involvement in the NEM effects, they are compatible with the argument that NEM and Gpp(NH)p act via the same

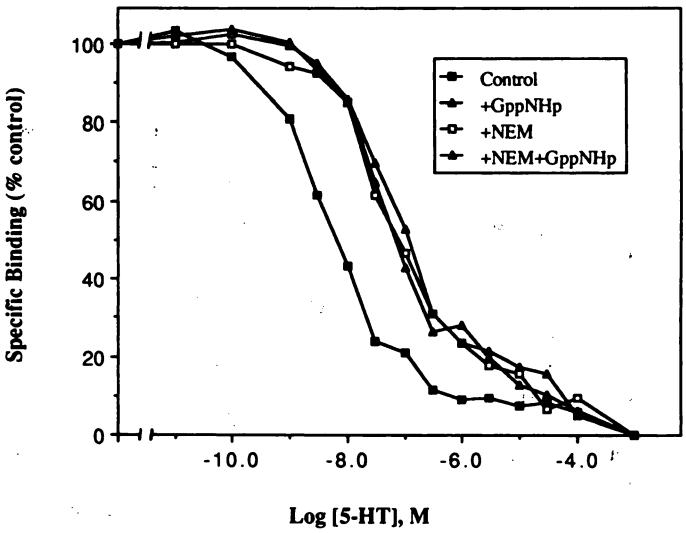

Fig. 7. Effects of NEM and Gpp(NH)p on competition of [^{125}I]ICYP binding by unlabeled 5-HT. Membranes from rat brain were preincubated in the absence or presence of 1 mM NEM at 30° for 15 min. [^{125}I]ICYP binding (0.84 nM) was then carried out in the presence of increasing concentrations of 5-HT, as indicated. Data are presented as per cent control specific binding. *Symbols* are defined in *inset*. $K_{d(app)}$ values from LIGAND analysis (one-site fit) are as follows: *Control*, membranes preincubated with buffer, 2.4 nM; *+GppNHp*, membranes preincubated with buffer, assayed in the presence of 0.1 mM Gpp(NH)p, 50 nM; *+NEM*, membranes preincubated with NEM, 109 nM; *+NEM+GppNHp*, membranes preincubated with NEM, assayed in the presence of Gpp(NH)p, 62 nM. Data shown are from a representative experiment; *points* represent means of triplicate samples, which varied by not more than 10%.

mechanism, namely causing dissociation of G protein from receptor and thereby shifting it into a lower affinity conformation for agonist binding.

In order to determine whether NEM inactivation of high affinity [^{3}H]5-HT binding is due to an interference with the endogenous receptor-G protein linkage, purified G proteins (G$_i$

and G_o) were added back to control and NEM-treated membranes. As shown in Fig. 8A, a significant portion of high affinity [^{3}H]5-HT binding was restored to NEM-treated membranes after addition of G proteins. Binding to control membranes was slightly enhanced by G protein addition. Gpp(NH)p addition resulted in decreased binding to control and G protein-reactivated membrane preparations, indicating that the increased binding was due to the addition of the G proteins. When analogous experiments were carried out under the site-specific conditions (Fig. 8, B and C), it was found that NEM inactivation of agonist binding to 5-HT_{1A} and 5-HT_{1D} binding sites could be reversed by addition of the G proteins. In contrast, binding to 5-HT_{1C} sites was not significantly increased by addition of the purified G proteins (data not shown).

In order to examine G protein sensitivity of the 5-HT_{1B} binding site, competition experiments similar to that described in Fig. 7 were carried out with addition of purified G protein to all conditions. That is, membranes were treated with buffer or NEM and washed extensively, and purified G proteins (G_i and G_o) were added to half of the samples (Fig. 9). Competition of [^{125}I]ICYP by unlabeled 5-HT was measured. As before, NEM treatment of membranes shifted the competition curve to the right. Addition of G proteins to NEM-treated membranes resulted in a leftward shift of the competition curve, indicating a restoration of high affinity binding. Although the curves appear nearly superimposable, the LIGAND-computed $K_{d(app)}$ of the G protein-reconstituted high affinity binding is not equal to that of control membranes (control, 1.8 nM; NEM plus G protein, 5 nM). This is probably due to the presence of a very low affinity (micromolar range) component of the competition curve, detected by LIGAND analysis for all curves, but of sufficient magnitude to be considered significant ($p < 0.05$) for a two-site fit of the data in only the two conditions in which G protein was added back to NEM-treated membranes (see legend to Fig. 9). When the low affinity site is separated out, the high affinity binding $K_{d(app)}$ is 2.9 nM for NEM-treated membranes to which G_i plus G_o was added. The effect of G protein addition is reversed by addition of Gpp(NH)p to the preparation, indicating that the restored high affinity binding is G protein dependent. Addition of G protein to control membranes did not affect the $K_{d(app)}$. These results suggest that the 5-HT_{1B} binding site is endogenously G protein linked.

Discussion

The sulfhydryl reagents NEM and PCMB inactivate binding of [^{3}H]5-HT to high affinity binding sites in rat and bovine brain membranes. This inactivation can be prevented by prior addition of DTT to the membranes (Fig. 1), suggesting that these reagents are exerting their effects via modification of sulfhydryl groups associated with the [^{3}H]5-HT binding sites. The two compounds react with sulfhydryl groups in different manners; NEM is a reasonably selective alkylating agent for sulfhydryl moieties, whereas PCMB is an organic mercurial compound that combines with thiols to form mercaptides. Our result is in contrast to that of an earlier study that reported that NEM inactivates [^{3}H]5-HT binding sites but that DTT does not protect against this inactivation (18). In our experiments, we found that the NEM reaction is quite rapid, even at 0°, and for this reason, the order of reagent addition is very important to the outcome of the experiment.[1]

Based on the observation that a portion of high affinity [^{3}H]5-HT binding is insensitive to NEM, we examined the NEM sensitivity of the various currently defined 5-HT receptor subtypes. Whereas binding of agonists to 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} subtypes is sensitive to NEM (Fig. 2), binding to the 5-HT_{1C} subtype (Figs. 2 and 4) and to the 5-HT_2 binding site (Fig. 5) is relatively insensitive to the reagent. 5-HT_{1C} binding sites comprise approximately 25–40% of high affinity [^{3}H]5-HT binding in bovine frontal cortex and 15–25% of high affinity [^{3}H]5-HT binding in rat cortical preparations.[2] These proportions are consistent with the fraction of high affinity [^{3}H]5-HT binding that is NEM-resistant in these species (Fig. 1).

Evidence for G protein and G protein-associated effector system involvement with central nervous system 5-HT recep-

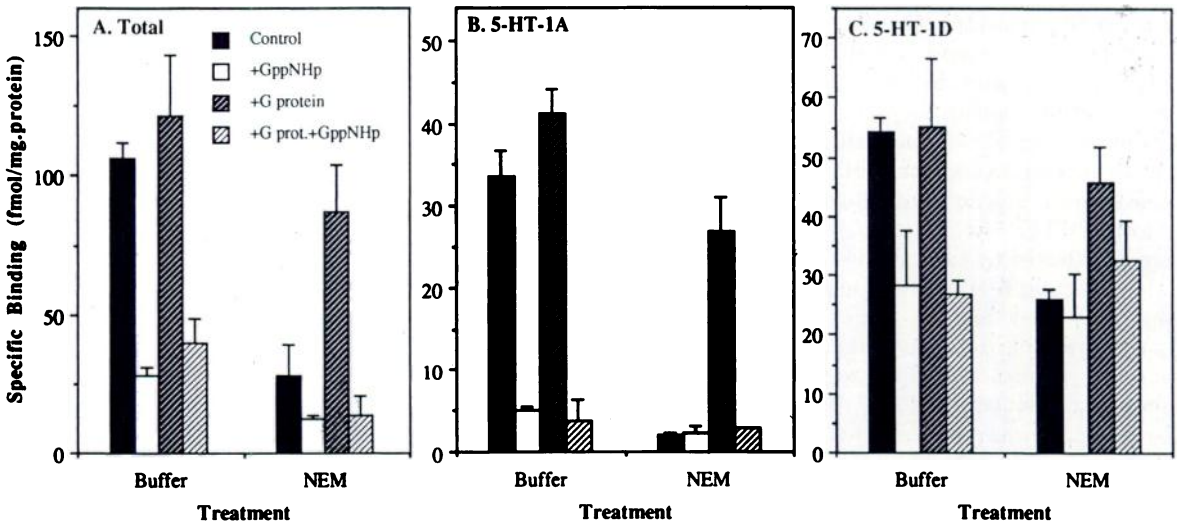

Fig. 8. Reconstitution of NEM-inactivated high affinity [^{3}H]5-HT binding by addition of G proteins. Membranes were treated with buffer or NEM, as indicated, then washed with DTT and buffer and mixed with purified G proteins (G_i plus G_o) as described in Materials and Methods. A, Binding of [^{3}H]5-HT (2.2–2.5 nM) to rat membranes, in the absence of masking agents, to all high affinity [^{3}H]5-HT binding sites. B, Binding of [^{3}H]DPAT to rat membranes, to 5-HT_{1A} binding sites. C, Binding of [^{3}H]5-HT to 5-HT_{1D} binding sites in bovine membranes in the presence of unlabeled DPAT and mesulergine. For all conditions, binding was assessed in the absence or presence of added G proteins and in the absence or presence of 100 μM Gpp(NH)p, as indicated in the *inset* legend to A. Data *bars* represent mean ± range of two separate experiments, each carried out in triplicate.

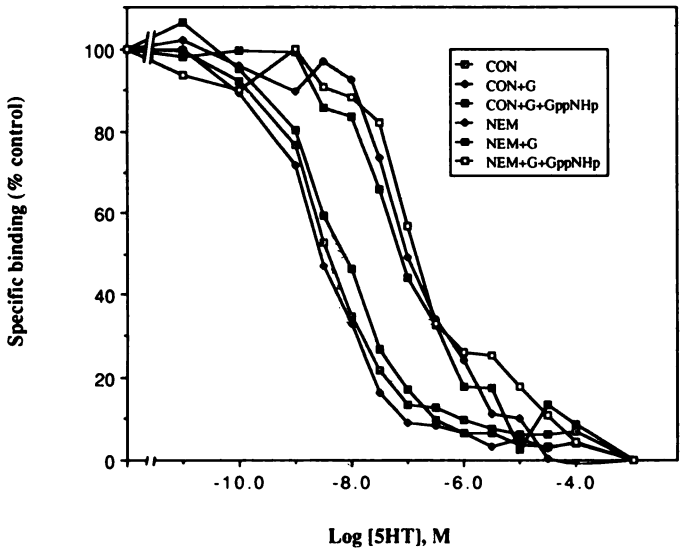

Fig. 9. Effects of NEM treatment and G protein addition on competition of [^{125}I]ICYP by 5-HT. Rat membranes were treated with buffer or NEM, washed with DTT and buffer, and mixed with buffer or G proteins as described in Materials and Methods. *Symbols* are defined in *inset*. Competition of 0.85 nM [^{125}I]ICYP binding by unlabeled 5-HT was assayed under the conditions listed, $K_{d(app)}$ of 5-HT(LIGAND) best fit, $p < 0.01$, one- or two-site as indicated as follows: *CON*, buffer-treated membranes, one-site 1.8 nM; *CON+G*, buffer-treated membranes with added G proteins, one-site 2.2 nM; *CON+G+GppNHp*, buffer-treated membranes with added G proteins and Gpp(NH)p (100 μM) in assay, one-site 29.8 nM; *NEM*, NEM-treated membranes, one-site 56.8 nM; *NEM+G*, NEM-treated membranes with added G proteins, one-site 5.1 nM, two-site ($p < 0.005$) 2.9 nM and 23.3 μM; *NEM+G+GppNHp*, NEM-treated membranes with added G proteins and Gpp(NH)p (100 μM) in assay, one-site, 88 nM, two site ($p < 0.05$) 21.0 nM and 6.2 μM. Data shown are from representative experiment. *Points* are means of triplicate samples, which were within 10% accordance and are expressed as per cent control specific binding.

tors has until recently been controversial. In early studies, Peroutka *et al.* (38) noted that, although addition of GTP to the binding assay caused a shift to the right of the binding curve, it did so only at much higher concentrations than reported for other binding sites. On this basis, these authors suggested that serotonin receptors had only a weak coupling with adenylate cyclase. More recently, the recognition of the coexistence of multiple high affinity [^{3}H]5-HT binding sites in most regions of the central nervous system and the emergence of several more specific ligands have allowed investigators to begin to unravel this problem. Blurton and Wood (39) reported that binding to 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1C}$ sites present in rat brain are all affected by coincubation with Gpp(NH)p but that only the effect on 5-HT$_{1A}$ sites require the presence of calcium ions. They used this as evidence to suggest that only the 5-HT$_{1A}$ sites are linked to adenylate cyclase. This finding is consistent with reports of 5-HT$_{1A}$ regulation of adenylate cyclase in either a stimulatory (40, 41) or inhibitory fashion (42, 43). Recently, however, Andrade *et al.* (44) have presented electrophysiological evidence that the 5-HT$_{1A}$ receptor is linked to a calcium-gated potassium channel in rat hippocampus, probably via a G protein, and that cyclic AMP is not involved as an intermediary in the gating mechanism. This may indicate that the 5-HT$_{1A}$ receptor can be linked to more than one type of effector system, possibly via different G proteins.

The biochemical linkages of 5-HT$_{1B}$ and 5-HT$_{1D}$ sites remain more elusive. There are some reports that 5-HT$_{1B}$ sites are presynaptic and thus regulate serotonin release (45, 46), although others have reported no diminution of binding to these sites after destruction of 5-HT-containing nerve terminals with 5,7-dihydroxytryptamine (39). Hamblin *et al.* (21) have recently shown that 5-HT$_{1B}$ sites exist in two discrete agonist affinity states regulated by GTP. This strongly supports G protein coupling for this binding site. Even less is known about 5-HT$_{1D}$ sites, except that they appear to predominate in bovine, human, and porcine brain, where few if any 5-HT$_{1B}$ sites are detectable (15). Rat brain, on the other hand, has a mixture of 5-HT$_{1B}$ and 5-HT$_{1D}$ sites in addition to 5-HT$_{1A}$ and 5-HT$_{1C}$ sites. 5-HT$_{1B}$ and 5-HT$_{1D}$ sites have somewhat similar pharmacological binding profiles and are sensitive to guanine nucleotides[2] (32).

In the current study, several lines of evidence are presented that suggest that the sulfhydryl group(s) responsible for inactivation of high affinity [^{3}H]5-HT binding are G protein-associated. First, the NEM-sensitive sulfhydryl group appears to be located distant from the ligand binding sites (Fig. 6). Second, at the 5-HT$_{1B}$ site, agonist binding is sensitive to NEM, whereas antagonist binding is not (Figs. 2 and 3). After NEM treatment of membranes, competition of antagonist binding to the 5-HT$_{1B}$ site by 5-HT is no longer sensitive to Gpp(NH)p (Fig. 7). Finally, we were able to reconstitute high affinity [^{3}H]5-HT binding to 5-HT$_{1A}$ and 5-HT$_{1D}$ sites and high affinity 5-HT competition of [^{125}I]ICYP binding to 5-HT$_{1B}$ sites by addition of G proteins (Figs. 8 and 9). These data are consistent with a system in which NEM irreversibly uncouples a G protein from the receptor to induce a decreased affinity for agonist but not antagonist binding. This uncoupling is most likely due to alkylation of a cysteine-residue near the ADP-ribosylation site of the endogenous α subunit as has been demonstrated by Winslow *et al.* (47) for the purified α subunit of G$_o$. The molecules containing the binding sites were apparently unaffected or only slightly affected by NEM, inasmuch as nearly all binding was restored by addition of the G proteins.

In the current study, we used a mixture of G$_i$ and G$_o$ (G$_{i/o}$) proteins for reconstituting binding. In preliminary studies, we have found that this combination or G$_o$ alone is more effective than G$_s$ in reconstitution of [^{3}H]DPAT binding to the 5-HT$_{1A}$ site.[4] G$_i$ and G$_o$ are equipotent in reconstituting high affinity binding to GABA-B binding sites (48) and purified muscarinic receptors from brain (49). Such results are compatible with the hypothesis that a given receptor can interact with more than one G protein and, potentially, with more than one effector system.

The observation that both 5-HT$_{1C}$ and 5-HT$_2$ binding sites are insensitive to NEM inhibition is particularly interesting in light of their other similarities. In rat, the two binding sites have remarkably similar drug displacement profiles, differing chiefly in their relative affinities for 5-HT, spiperone, and, to some degree, cinanserin and ketanserin (15). Moreover, both receptors have also been shown to be linked to PI turnover (50–53). In the case of the 5-HT$_2$ receptor, evidence from binding studies indicates that this linkage is mediated via a G protein (54). Although there is some evidence that G proteins also link receptors to PI turnover, the characteristics of the G proteins involved in different physiological processes vary greatly. That is, *Bordatella pertussis* toxin, which ADP-ribosylates and thus inactivates G$_i$ as well as G$_o$, has been shown to

[4] C. A. Stratford and R. D. Ciaranello, unpublished observations.

interfere with receptor-linked PI turnover in some cases but not in others (see Refs. 55 and 56 for reviews). There has been some speculation that, in brain, the highly prevalent G_o species might mediate PI turnover. The evidence presented here militates against that notion, in the case of 5-HT_{1C} and 5-HT_2 receptors, because the α subunit of the G_o species [termed α_{39} by Neer and co-workers (47)] is susceptible to inactivation by NEM alkylation. Our data indicate that the G protein(s) that mediate 5-HT_{1C} and 5-HT_2 signal transduction are different than those that link to 5-HT_{1A}, 5-HT_{1B}, or 5-HT_{1D} binding sites.

In summary, in the current study we have presented nonpharmacological evidence for heterogenity between high affinity [^{3}H]5-HT bindings sites in rat and bovine brain. We suggest that the NEM inhibition of agonist binding to 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} binding sites are conferred upon these sites via linkage to NEM-sensitive G proteins.

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