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# Relationship of Mu Opioid Receptor Binding to Activation of G-Proteins in Specific Rat Brain Regions

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**ABSTRACT.** This study investigated the relationship between mu receptor binding and mu agonist activation of G-proteins in the rat brain. To directly compare agonist potencies in receptor binding ($K_i$ values) and G-protein activation ($K_s$ values), both agonist-stimulated [$^{35}$S]guanosine-5′-*O*-(γ-thio)-triphosphate ([$^{35}$S]GTPγS) and [$^{3}$H]naloxone binding assays were conducted under identical conditions, using the full mu agonist [d-Ala$^2$, *N*-Me$^4$, Gly$^5$-ol]-enkephalin (DAMGO). DAMGO exhibited biphasic competition of [$^{3}$H]naloxone binding and stimulation of [$^{35}$S]GTPγS binding in most regions. Whereas the high-affinity component represented a large percentage (50–80%) of total receptor sites, the high-affinity component of DAMGO-stimulated [$^{35}$S]GTPγS binding was much lower, <30% of the total, and in most regions significant stimulation of [$^{35}$S]GTPγS binding did not occur until the high-affinity binding sites were completely occupied. Moreover, the low-affinity potencies for DAMGO in receptor binding and G-protein activation were the same across different regions. Receptor–transducer amplification factors were calculated by the ratio of the apparent $B_{max}$ of net agonist-stimulated [$^{35}$S]GTPγS binding to the $B_{max}$ of receptor binding. Amplification factors for the nine regions examined were relatively high and varied significantly across regions, from a ratio of 8 in the thalamus to 38 in the cortex, suggesting that the efficiency of mu opioid receptor coupling to G-proteins varies across brain regions. BIOCHEM PHARMACOL **59**;11:1395–1401, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** mu opioid; G-protein; agonist efficacy; [$^{35}$S]GTPγS; naloxone; signal transduction

Opiate alkaloids exert their biological actions by binding to mu opioid receptors, which belong to the superfamily of seven transmembrane spanning receptors that couple to G-proteins [1, 2]. Agonist activation of the mu opioid receptor causes inhibition of adenylyl cyclase [3, 4], stimulation of potassium channel conductance [5, 6], and inhibition of calcium channel conductance [7, 8] via pertussis toxin-sensitive $G_i/G_o$ proteins [9, 10]. For many G-protein-coupled receptors, the efficacies of agonists in activating G-proteins can be determined in binding assays using the hydrolysis-resistant GTP analog [$^{35}$S]GTPγS§ in the presence of excess GDP [11–13]. This assay was established for mu opioid receptors by Traynor and Nahorski [14], who demonstrated that mu agonists of varying efficacies differentially stimulated [$^{35}$S]GTPγS binding to SH-SY5Y cell membranes. Studies from our laboratory [15, 16] have further elucidated mechanisms of full and partial agonist efficacies in stimulating [$^{35}$S]GTPγS binding in a variety of cell systems and in the rat brain. These experiments showed that in brain membranes, the mu selective opioid peptide analog DAMGO was a full agonist, morphine was a high-efficacy partial agonist, and buprenorphine was a low-efficacy partial agonist. This was in contrast to stably transfected CHO cells that overexpress mu receptors, where both DAMGO and morphine were full agonists and buprenorphine was a moderate-efficacy partial agonist. Comparison of agonist potencies to receptor binding potencies suggested that these relative efficacy differences were due to the low receptor reserve in the brain compared with that of the transfected CHO cells [15].

Other studies from our laboratory have explored the brain regional specificity of opioid activation of G-proteins using [$^{35}$S]GTPγS autoradiography [17, 18]. Although [$^{35}$S]GTPγS autoradiography provides a high degree of neuroanatomical resolution, it is difficult to quantitatively explore the relationship between opioid receptor binding and opioid agonist activation of G-proteins. For example, the potencies of agonists in stimulating [$^{35}$S]GTPγS binding in brain membranes are usually several orders of magnitude lower than the corresponding potencies in traditional receptor binding assays [15, 19]. Most of this

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§ *Abbreviations:* GTPγS, guanosine-5′-*O*-(γ-thio)-triphosphate; and DAMGO, [D-Ala [2], *N*-Me$^4$, Gly [5]-ol]-enkephalin.

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difference is caused by the use of sodium and guanine nucleotides in the [$^{35}$S]GTPγS binding assay, which shift agonist binding into low-affinity states [20, 21]. To directly compare agonist potencies in receptor binding and G-protein activation, both assays must be performed under the same conditions, which necessitates the use of a labeled antagonist as a radioligand to provide high-affinity receptor binding in the presence of sodium and guanine nucleotides. The present study adapted these assays to determine whether the relationship between receptor occupancy and G-protein activation varies across brain regions.

In addition, by using a combination of [$^{35}$S]GTPγS autoradiography and membrane studies, previous studies calculated the receptor–transducer amplification factor for mu receptors in rat striatal membranes by comparing the apparent $B_{max}$ of [$^{35}$S]GTPγS binding and the $B_{max}$ of receptor binding [22]. Those studies showed that the amplification factor for mu receptors was relatively high, and suggested that mu receptors catalytically activated approximately 20 G-proteins per receptor in the rat striatum. In addition to exploring differences in agonist potencies, the present study used the same techniques to examine whether there were differences in the amplification factor for mu receptors across several rat brain regions.

## MATERIALS AND METHODS

### *Materials*

Male Sprague–Dawley rats (150–200 g) were purchased from Zivic Miller. [$^{35}$S]GTPγS (1250 Ci/mmol) and [$^{3}$H]naloxone (57.5 Ci/mmol) were purchased from the New England Nuclear Corp. Guanosine-5′-diphosphate and GTPγS were obtained from Boehringer Mannheim. DAMGO, naloxone, naltrindole, and adenosine deaminase were purchased from the Sigma Chemical Co. Ecolite scintillation fluid was purchased from Fisher Scientific. All other reagent grade chemicals were purchased from Sigma or Fisher Scientific.

### *Membrane Preparations*

Rats were euthanized by decapitation, and nine brain regions (amygdala, brainstem, colliculus, frontal cortex, hippocampus, hypothalamus, sensomotor cortex, striatum, and thalamus) were dissected on ice and homogenized with a Tissumizer (Tekmar) in cold membrane buffer (50 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EGTA). The homogenate was diluted to 30 mL with membrane buffer and centrifuged at 48,000 *g* for 10 min at 4°. The supernatant was discarded, and the pellets were resuspended in membrane buffer, homogenized, and centrifuged as before. After the second centrifugation, the supernatant was discarded, and the pellet was resuspended in cold assay buffer (50 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl). Then the membranes were assayed for protein content [23], and aliquots were stored at −80°.

### *[$^{3}$H]Naloxone and [$^{35}$S]GTPγS Binding Assays*

Assays for [$^{35}$S]GTPγS and [$^{3}$H]naloxone binding were designed so that the conditions were virtually identical for both assays. Membranes were thawed, homogenized in 10 mL of assay buffer, and incubated at 30° for 10 min with 4 mU/mL of adenosine deaminase. Then membranes were diluted to 30 mL with assay buffer and assayed for protein content [23]. Both [$^{3}$H]naloxone and [$^{35}$S]GTPγS assays included 0.5 nM naltrindole (sufficient to block delta opioid receptors without affecting mu receptor sites) and 10 μM GDP in assay buffer in a final volume of 1 mL. For DAMGO concentration–effect analyses, [$^{3}$H]naloxone and [$^{35}$S]GTPγS assays were conducted with 0.3 nM to 50 μM DAMGO. For [$^{35}$S]GTPγS binding, membranes (5–15 μg, depending on the brain region) were incubated for 2 hr at 30° with 0.05 nM [$^{35}$S]GTPγS and 1.2 nM unlabeled naloxone. For [$^{3}$H]naloxone binding, membranes (90–120 μg) were incubated for 2 hr at 30° with 1.2 nM [$^{3}$H]naloxone and 0.05 nM unlabeled GTPγS. For GTPγS saturation analysis, membranes were incubated with 10 μM DAMGO, 10 μM GDP, 0.5 to 20 nM unlabeled GTPγS, and 0.1 nM [$^{35}$S]GTPγS in assay buffer. For [$^{3}$H]naloxone saturation analysis, membranes were incubated with 0.5 to 10 nM [$^{3}$H]naloxone, 10 μM GDP, 0.5 nM naltrindole, and 0.05 nM unlabeled GTPγS. For both assays, reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with 3 mL of cold 50 mM Tris–HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [$^{35}$S]GTPγS and 42% efficiency for [$^{3}$H]naloxone after overnight extraction of the filters in 4 mL of Ecolite scintillation fluid.

Nonspecific binding was determined with 10 μM GTPγS for [$^{35}$S]GTPγS binding or 10 μM naloxone for [$^{3}$H]naloxone binding. For [$^{35}$S]GTPγS assays, basal binding was determined in the absence of DAMGO; net agonist-stimulated [$^{35}$S]GTPγS binding was calculated by subtracting basal binding from total agonist-stimulated binding.

### *Data Analysis*

Analyses of agonist concentration–effect curves, receptor binding, and saturation curves were conducted by nonlinear regression with the use of JMP for Macintosh (SAS Institute). Following the nonlinear curve fitting to a two-site model, $K_i$ values for DAMGO in receptor binding assays were calculated from corresponding $IC_{50}$ values using the Cheng–Prusoff equation [24], with 1.8 nM as the calculated $K_D$ value for naloxone. Likewise, in [$^{35}$S]GTPγS assays, $EC_{50}$ values from DAMGO were corrected to apparent $K_s$ values with the Cheng–Prusoff equation to correct for the presence of naloxone in the [$^{35}$S]GTPγS assay, using 1.8 nM as the $K_D$ for naloxone. These corrections can be done in this way because naloxone binds monophasically to mu opioid receptors, with a single $K_D$ value in receptor

binding and a single $K_i$ value in blocking mu agonist-stimulated [$^{35}$S]GTPγS binding. Mean amplification factors were calculated by dividing mean net agonist-stimulated [$^{35}$S]GTPγS binding apparent $B_{max}$ values by mean receptor binding $B_{max}$ values. The standard error of each amplification factor was calculated as the square root of the variance as estimated by the equation:

$$\mathrm{Var}\left(\frac{x1}{x2}\right) = \frac{(s1)^2}{(x2)^2} + \frac{(x1)^2 \times (s2)^2}{(x2)^4}$$

where x1 is the mean $B_{max}$ value for G-proteins, x2 is the mean $B_{max}$ value for receptors, and s1 and s2 are the respective $B_{max}$ standard errors [25]. Significant differences in regional amplification factors were determined by multiple Student's *t*-tests to compare each region with every other region at a significance level of $\alpha = 0.0045$ (Bonferroni adjustment to $\alpha = 0.05$ for 9 groups). Significant differences ($P < 0.05$) between other values were determined with JMP to perform Student's *t*-tests for two groups, or ANOVA and the Tukey–Kramer HSD test for multiple comparisons. Significant differences are indicated in the figures and tables by letters: values that are not different are indicated by the same single letter. Data are reported as means ± SEM of at least three separate experiments, each performed in triplicate, unless otherwise indicated.

## RESULTS

Several binding assays were used to compare the parameters of mu opioid receptor binding and mu opioid activation of G-proteins in nine rat brain regions: amygdala, brainstem, colliculus, frontal cortex, hippocampus, hypothalamus, sensomotor cortex, striatum, and thalamus. First, the potency (G-$K_s$) and the efficacy (G-$E_{max}$) of DAMGO in stimulating [$^{35}$S]GTPγS binding to membranes were measured by constructing DAMGO concentration–effect curves. Second, DAMGO potencies in receptor binding (R-$K_i$) values were calculated by competition of [$^3$H]naloxone binding. Third, the ability of DAMGO to increase the affinity of the G-protein for [$^{35}$S]GTPγS (G-$K_D$) and to stimulate a maximal number of G-proteins (G-$B_{max}$) was determined by net agonist-stimulated GTPγS saturation binding analysis. Fourth, [$^3$H]naloxone saturation analyses were used to measure mu opioid receptor density (R-$B_{max}$). To compare all these parameters directly, both [$^{35}$S]GTPγS and receptor binding assays were conducted under identical conditions, which included the same drugs, buffers, membrane preparations, and incubation conditions. For this reason, maximal stimulation of [$^{35}$S]GTPγS binding by DAMGO was somewhat lower than reported previously [16].

### *DAMGO Potencies in Stimulating [$^{35}$S]GTPγS Binding and Displacing [$^3$H]Naloxone Binding*

When incubated in the presence of 10 μM GDP, DAMGO markedly increased the binding of [$^{35}$S]GTPγS to membranes from the nine rat brain regions in a concentration-dependent and saturable manner. Figure 1 illustrates the concentration–effect curves for DAMGO in stimulating [$^{35}$S]GTPγS binding and displacing [$^3$H]naloxone binding in four of the rat brain regions. A visual inspection of these curves reveals the general finding that DAMGO competition of [$^3$H]naloxone binding occurred at lower concentrations than those required for agonist activation of G-proteins. This finding is particularly evident in thalamus and brainstem. For example, in thalamic membranes, the concentration of DAMGO that inhibited 50% of [$^3$H]naloxone binding (approx. 30 nM) stimulated less than 15% of maximal agonist-stimulated [$^{35}$S]GTPγS binding. Similarly, in brainstem membranes, the concentration of DAMGO that inhibited 50% of [$^3$H]naloxone binding (approx. 50 nM) stimulated approximately 20% of maximal agonist-stimulated [$^{35}$S]GTPγS binding.

The potency of DAMGO was calculated in both [$^3$H]naloxone competition assays and [$^{35}$S]GTPγS assays (Table 1). In both assays, DAMGO concentration–effect curves exhibited Hill slopes significantly less than 1 (not shown) and were best fit by biphasic analysis in all regions. Analysis of the [$^3$H]naloxone competition curves (Table 1) revealed high-affinity R-$K_i$ values between 1 and 7 nM, and low-affinity R-$K_i$ values between 900 and 3300 nM. In all cases, the percentage of high-affinity R-$K_i$ sites was significant, between 40 and 80% of total sites. In contrast, for G-$K_s$ values of DAMGO-stimulated [$^{35}$S]GTPγS binding, the percentage of high-affinity [$^{35}$S]GTPγS binding sites was less than 30% in every region (Table 1). Thus, although the Hill slopes for these curves were all less than 1, many high-affinity G-$K_s$ values were not reliable and, therefore, were not reported in Table 1. However, across the regions, the average high-affinity $K_s$ value was 4.9 ± 1.4 nM, which was comparable to the mean high-affinity R-$K_i$ value (average of all R-$K_i$ values in Table 1) of 3.5 ± 0.8 nM. Analysis of low-affinity G-$K_s$ values for DAMGO was more reliable across regions, providing values that varied between 890 and 3200 nM. In each region, none of the low-affinity G-$K_s$ values were significantly different from the corresponding low-affinity R-$K_i$ values ($P > 0.17$). Indeed, the ratios between low-affinity R-$K_i$ and G-$K_s$ values (not shown) ranged from 0.5 to 1.4, none of which were significantly different from 1.

### *Agonist Efficacies and Receptor–Transducer Amplification*

To quantify regional differences in agonist efficacy, DAMGO concentration–effect curves in stimulating [$^{35}$S]GTPγS binding were analyzed for maximal stimulation (G-$E_{max}$) values (Table 2). Maximal stimulation of [$^{35}$S]GTPγS binding by DAMGO varied from 39% in the striatum to 98% in the thalamus, with significant differences between regions. DAMGO displayed relatively low efficacy in stimulating [$^{35}$S]GTPγS binding in striatum, moderate efficacy in the sensomotor cortex, hippocampus,

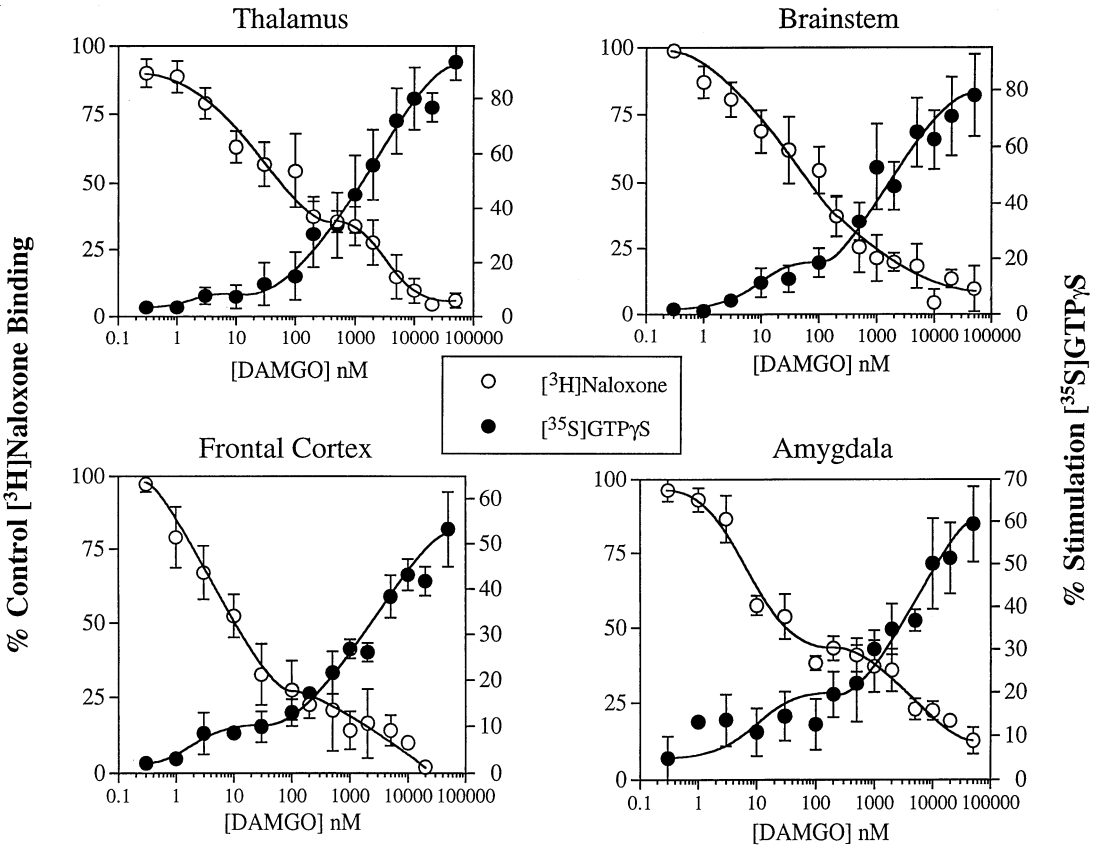


**FIG. 1. Concentration–effect curves of DAMGO for stimulation of [$^{35}$S]GTPγS binding and inhibition of [$^{3}$H]naloxone binding in four rat brain regions (thalamus, brainstem, frontal cortex, and amygdala). Both [$^{35}$S]GTPγS and [$^{3}$H]naloxone binding assays were conducted under identical conditions as described in Materials and Methods. Data are presented as means ± SEM from 3–5 separate experiments.**

frontal cortex, amygdala, hypothalamus, colliculus, and brainstem, and relatively high efficacy in the thalamus.

Amplification between receptors and transducers can be estimated by the ratio between the maximal number of agonist-stimulated G-proteins and the number of receptor binding sites. Mu opioid receptor density (R-$B_{max}$) was determined with [$^{3}$H]naloxone saturation analysis in all nine rat brain regions (Table 2). The lowest R-$B_{max}$ value was found in the sensomotor cortex (127 fmol/mg), while the thalamus displayed the highest R-$B_{max}$ (511 fmol/mg). Other than the fact that the R-$B_{max}$ value in the thalamus was significantly different from those of all other regions and the R-$B_{max}$ values in the hypothalamus and amygdala were significantly different from that of the sensomotor

**TABLE 1. Biphasic analysis of DAMGO potency in inhibiting [$^{3}$H]naloxone and stimulating [$^{35}$S]GTPγS binding in various rat brain regions**

| | [$^{35}$S]GTPγS binding | | [$^{3}$H]Naloxone binding | | |
|---|---|---|---|---|---|
| Region | % High G-$K_s$ | G-$K_s$ (Low) (nM) | % High R-$K_i$ | R-$K_i$ (High) (nM) | R-$K_i$ (Low) (nM) |
| Amygdala | 15 ± 6 | 1670 ± 590 | 55 ± 11 | 2.68 ± 1.8 | 2330 ± 820 |
| Brainstem | 28 ± 8 | 2710 ± 1340 | 46 ± 7 | 6.95 ± 4.3 | 1340 ± 550 |
| Colliculus | 12 ± 6 | 1610 ± 550 | 38 ± 8 | 1.90 ± 1.1 | 2010 ± 1350 |
| Frontal cortex | 13 ± 2 | 1650 ± 660 | 77 ± 10 | 1.68 ± 1.7 | 2250 ± 900 |
| Hippocampus | 9 ± 3 | 3200 ± 970 | 53 ± 7 | 6.90 ± 2.8 | 1680 ± 630 |
| Hypothalamus | 24 ± 5 | 2790 ± 450 | 81 ± 2 | 2.44 ± 0.1 | 2630 ± 1150 |
| Sensomotor cortex | 11 ± 2 | 2690 ± 1080 | 65 ± 6 | 1.19 ± 0.6 | 3270 ± 200 |
| Striatum | 9 ± 2 | 3120 ± 1430 | 70 ± 3 | 2.32 ± 0.5 | 1790 ± 720 |
| Thalamus | 9 ± 6 | 890 ± 390 | 54 ± 12 | 5.19 ± 1.2 | 860 ± 380 |

G-$K_s$ values were calculated from DAMGO-stimulated [$^{35}$S]GTPγS binding, while R-$K_i$ values were calculated from DAMGO inhibition of [$^{3}$H]naloxone binding as described in Materials and Methods. The columns "% High" refer to the percentage of high-affinity sites as calculated from nonlinear fitting. Data represent means ± SEM from concentration–effect curves conducted in triplicate three or more times in membranes from each brain region.

**TABLE 2. [$^3$H]Naloxone and [$^{35}$S]GTPγS binding parameters**

| Region | G-$E_{max}$ (%) | G-$K_D$ (nM) | G-$B_{max}$ (pmol/mg) | R-$B_{max}$ (pmol/mg) | G-$B_{max}$/R-$B_{max}$ |
|---|---|---|---|---|---|
| Amygdala | 59.4 ± 8.9$^{bc}$ | 1.90 ± 0.52 | 4.48 ± 1.00 | 0.240 ± 0.022$^{a}$ | 18.6 ± 4.5$^{bc}$ |
| Brainstem | 78.1 ± 14.6$^{ab}$ | 2.33 ± 0.18 | 4.09 ± 0.32 | 0.192 ± 0.028$^{ab}$ | 21.3 ± 3.5$^{bc}$ |
| Colliculus | 76.3 ± 5.2$^{ab}$ | 1.26 ± 0.33 | 6.15 ± 1.20 | 0.208 ± 0.023$^{ab}$ | 29.6 ± 6.4$^{cd}$ |
| Frontal cortex | 53.2 ± 8.3$^{bc}$ | 1.68 ± 0.27 | 3.83 ± 0.53 | 0.195 ± 0.019$^{ab}$ | 19.6 ± 3.3$^{bc}$ |
| Hippocampus | 49.6 ± 1.9$^{bc}$ | 3.00 ± 0.16 | 5.49 ± 0.67 | 0.188 ± 0.016$^{ab}$ | 30.3 ± 4.5$^{d}$ |
| Hypothalamus | 75.6 ± 4.2$^{ab}$ | 1.35 ± 0.07 | 4.94 ± 0.16 | 0.261 ± 0.023$^{a}$ | 19.0 ± 1.8$^{bc}$ |
| Sensomotor cortex | 42.5 ± 0.9$^{bc}$ | 2.78 ± 0.22 | 4.78 ± 0.44 | 0.127 ± 0.012$^{b}$ | 37.7 ± 4.9$^{d}$ |
| Striatum | 39.2 ± 7.8$^{c}$ | 1.24 ± 0.10 | 3.32 ± 0.41 | 0.181 ± 0.011$^{ab}$ | 18.3 ± 2.5$^{ab}$ |
| Thalamus | 98.5 ± 5.4$^{a}$ | 1.42 ± 0.14 | 4.12 ± 0.85 | 0.511 ± 0.028$^{c}$ | 8.1 ± 2.1$^{a}$ |

DAMGO maximal efficacy (G-$E_{max}$) values were determined from DAMGO-stimulated [$^{35}$S]GTPγS concentration curves in each rat brain region, and are expressed as percent stimulation. G-$B_{max}$ and G-$K_D$ values were determined from net DAMGO-stimulated [$^{35}$S]GTPγS saturation analyses. Mu receptor $B_{max}$ (R-$B_{max}$) values were determined from [$^3$H]naloxone saturation analyses. Data represent means ± SEM of experiments conducted in triplicate three or more times. Identical letter designations represent no significant difference between regions by Student's *t*-test, $P < 0.05$.

cortex, the R-$B_{max}$ values in the striatum, hippocampus, brainstem, frontal cortex, and colliculus were similar, ranging from 181 to 208 fmol/mg.

Saturation analysis of DAMGO-stimulated [$^{35}$S]GTPγS binding determined the apparent G-$B_{max}$ value of DAMGO-activated G-proteins in each region by varying the concentration of GTPγS in the presence and absence of agonist. G-$B_{max}$ values of DAMGO-activated G-proteins ranged from 3.3 pmol/mg in the striatum to 6.2 pmol/mg in the colliculus (Table 2). These experiments also showed that, in the presence of DAMGO, [$^{35}$S]GTPγS bound with high affinity, with G-$K_D$ values ranging from 1.2 to 3 nM in the nine rat brain regions investigated (Table 2).

The receptor–transducer amplification factor for each region was calculated by the ratio G-$B_{max}$:R-$B_{max}$ (Table 2). The amplification factors for mu opioid receptors in the rat brain regions examined were relatively high in all regions, a finding that agrees with previous data [22]. Amplification factors ranged from 8 to 38 in different brain regions, with three significantly different groups. The thalamus featured the lowest amplification factor of 8 G-proteins activated per mu receptor. Regions exhibiting moderate amplification values (18 to 21) included the striatum, amygdala, hypothalamus, frontal cortex, and brainstem, while the hippocampus, colliculus, and sensomotor cortex displayed relatively high amplification factors (29 to 38).

## DISCUSSION

The present study explored three questions about the relationship between mu opioid receptor binding and activation of G-proteins in brain membranes. First, what is the relationship between agonist potencies in binding to receptors versus their potencies in activating G-proteins? Second, does agonist potency in activating G-proteins vary across brain regions? Third, does the amplification factor between mu receptor number and mu agonist-stimulated G-proteins vary across brain regions? To answer these questions, both receptor and [$^{35}$S]GTPγS binding assays were conducted under the same conditions. Because sodium and guanine nucleotides shift agonist binding into low-affinity states [20], it is necessary to perform the receptor binding experiments with a labeled antagonist (in this case, [$^3$H]naloxone). To compare results from the receptor binding assays with those from [$^{35}$S]GTPγS assays, NaCl, GDP, and unlabeled GTPγS were added to the [$^3$H]naloxone assays to mimic the conditions of [$^{35}$S]GTPγS binding. Similarly, the [$^{35}$S]GTPγS assay contained the same concentration of naloxone used in the [$^3$H]naloxone assays. The delta opioid antagonist naltrindole was also added to block binding of ligands to delta receptors. Under these conditions, the $K_i$ value of naltrindole was 19 nM at mu receptors and 0.12 nM at delta receptors (Maher C and Childers S, unpublished observations). As assessed using the Langmuir equation, 0.5 nM naltrindole occupied approximately 2% of mu receptors, compared with 81% occupancy of delta receptors.

In the brain regions tested, several parameters varied across regions, while other parameters remained relatively constant. In [$^3$H]naloxone saturation assays, the $K_D$ values for naloxone did not vary across regions (data not shown), although the mu receptor binding $B_{max}$ varied significantly. Since naloxone is a purely neutral antagonist and binds equally well to both high- and low-affinity agonist receptor states, it is not surprising that its $K_D$ was constant. Similarly, in [$^{35}$S]GTPγS saturation analyses, $K_D$ values of DAMGO-stimulated GTPγS binding (a measure of the ability of an agonist to fully activate Gα) did not vary significantly between regions, indicating that DAMGO was a full mu agonist in all regions tested [16].

Other parameters, such as mu receptor $B_{max}$ values and the maximal efficacy of DAMGO-stimulated [$^{35}$S]GTPγS binding, varied significantly across regions. As expected, the mu receptor $B_{max}$ correlated well with the maximal efficacy (G-$E_{max}$) of DAMGO in stimulating [$^{35}$S]GTPγS binding ($r = 0.783$, $P = 0.013$). Previous studies from our laboratory [15, 26] showed that G-$E_{max}$ was best correlated with the ratio between the [$^{35}$S]GTPγS saturation parameters G-$B_{max}$ and G-$K_D$. In the current study, G-$E_{max}$ correlated relatively well with this ratio ($r = 0.766$, $P = 0.016$). Neither the correlation between G-$E_{max}$ and G-$K_D$

($r = 0.622$, $P = 0.07$) nor the correlation between G-$E_{max}$ and G-$B_{max}$ ($r = 0.283$, $P = 0.46$) alone was significant. These data, therefore, support the concept that agonist efficacy (G-$E_{max}$) is the result of both the activation of Gα by agonist (G-$K_D$) and the number of G-proteins activated (G-$B_{max}$) [16].

This study also compared potencies of DAMGO in binding to mu receptors and in activating G-proteins under the same conditions. Figure 1 shows that the relationship between receptor binding and G-protein activation was not 1:1; in fact, in some regions, >50% occupation of receptors by DAMGO was necessary before significant stimulation of [$^{35}$S]GTPγS binding was observed. Biphasic analysis revealed that most of these discrepancies arose from the high-affinity sites, where, in all cases, the percentages of high-affinity receptor sites (R-$K_i$) were much higher than the corresponding percentages of high-affinity G-$K_s$ values of DAMGO-stimulated [$^{35}$S]GTPγS binding. Thus, low-affinity mu receptor sites must be occupied to produce significant activation of G-proteins. The reason for this relationship is not clear, but since the high-affinity sites may be precoupled or spontaneously active receptors [27, 28], the occupation of these sites by agonist may not increase G-protein activity much above basal levels. As the agonist concentration is increased and the high-affinity sites become saturated, the agonist binds to the lower affinity "uncoupled" sites. The binding of agonist may then increase the affinity of the receptor for G-proteins, which subsequently causes the agonist-bound receptor to couple with and activate G-proteins.

The correlation between low-affinity agonist binding and low-affinity agonist activation of G-proteins parallels other functional opioid effects in the brain, including adenylyl cyclase [29] and ion channel function [6, 30], where agonist potencies are >100 nM. These relatively low potencies in brain are in contrast to the higher agonist potencies observed (<10 nM) in transformed cells, where large numbers of receptors create an effective receptor reserve and an increase in agonist potency [16, 31].

Since receptor reserve occurs when a relatively low occupancy of receptors by agonist produces maximal agonist efficacy, a significant receptor reserve is implied by a R-$K_i$:G-$K_s$ ratio greater than 1 [15, 32, 33]. Because of the low detectability of high-affinity G-$K_s$ sites, it was not possible to calculate the ratio between high-affinity receptor and activated G-protein sites; nevertheless, the finding that the average calculated high-affinity G-$K_s$ value, approximately 5 nM, was the same as the average R-$K_i$ value (4 nM) suggests that no significant receptor reserve exists at the high-affinity site. Similarly, for the low-affinity sites, where determination of values could be obtained with better reliability, there were no significant differences between low-affinity R-$K_i$ and G-$K_s$ values. Therefore, these data strongly suggest a lack of significant receptor/G-protein reserve at any site for mu receptors. It should be noted that these results do not preclude receptor reserve at sites further downstream (e.g. effectors) as previously described [34–36].

Previously, we reported that the amplification factor (the ratio between the receptor $B_{max}$ and the $B_{max}$ of agonist-activated G-proteins) for mu receptors in rat striatal membranes was quite high, approximately 20 [22]. The present study showed that amplification factors for DAMGO were different among rat brain regions, ranging from 8 to 38. The simple interpretation, that the amplification factor represents the number of G-proteins activated by a mu receptor, is not strictly correct because [$^{35}$S]GTPγS $K_D$ and $B_{max}$ values are only apparent values [25]. Therefore, these amplification factors are most useful when interpreted in a relative manner. The mechanisms responsible for the differences in receptor–G-protein amplification factors have not been elucidated yet. One possibility is that mu receptors activate different subtypes of Gα in different regions [37], or involve varying localization of G-protein β-γ subunits [38]; regional variations in the cellular composition of G-proteins thus could produce differences in receptor amplification. Another interpretation is that the relative uniformity of G-$B_{max}$ values across brain regions suggests that all available G-proteins in each region are being activated. If this were the case, then the amplification factor would be dependent not only on the number of mu receptors in each region but also on the amount of G-proteins able to be activated. Determining the mechanisms responsible for these differences will be the focus of future studies.

These regional differences in mu receptor efficiency may have important consequences in the physiological actions of opioids. Physiological effects of drugs may not always correlate with the number of receptors in appropriate brain regions, as calculated solely by receptor $B_{max}$ values. Assay of receptor-activated G-proteins provides a functional approach to this issue. In the brain, effects of mu agonists are complex, and differences in agonist-bound receptor–G-protein interactions may account for downstream differences in signal transduction. Accordingly, these variations in potency and efficacy at the level of receptor-activated G-proteins may contribute to the differential expression of behavioral effects of opiates.

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