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Effects of Chronic Morphine Administration on μ Opioid Receptor-Stimulated [^{35}S]GTPγS Autoradiography in Rat Brain

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Chronic opiate administration results in the development of tolerance and dependence, but the regulation of μ opioid receptor function during this process is not clearly understood. To localize changes in μ opioid receptor-coupled G-protein activity in various brain regions after chronic morphine treatment, the present study examined μ opioid agonist-stimulated [^{35}S]GTPγS binding to brain sections by *in vitro* autoradiography. Rats were treated for 12 d with increasing doses (10–320 mg · kg^{-1} · d^{-1}) of morphine. Control rats were injected with either saline or a single acute injection of morphine (20 mg/kg). μ opioid-stimulated [^{35}S]GTPγS binding was measured by autoradiography of brain sections in the presence and absence of the μ opioid-selective agonist DAMGO. In rats injected with a single acute dose of morphine, no significant changes were detected in basal or agonist-stimulated [^{35}S]GTPγS binding in any region. In sections from chronic morphine-treated rats, however, DAMGO-stimulated [^{35}S]GTPγS binding was reduced significantly compared with control rats in the following brainstem nuclei: dorsal raphe nucleus, locus coeruleus, lateral and medial parabrachial nuclei, and commissural nucleus tractus solitarius. No significant changes were observed in several other brain regions, including the nucleus accumbens, amygdala, thalamus, and substantia nigra. These data indicate that chronic morphine administration results in reductions in μ opioid activation of G-proteins in specific brainstem nuclei involved in physiological homeostasis and autonomic function, which may have implications in the development of opiate tolerance and physical dependence.

Key words: chronic morphine; [^{35}S]GTPγS autoradiography; μ opioid receptor; G-protein; dorsal raphe nucleus; nucleus locus coeruleus; nucleus tractus solitarius; parabrachial nucleus

Opioid receptors are coupled to G-proteins of the G_i/G_o family (Burns et al., 1983; Childers, 1991; Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993) and inhibit adenylyl cyclase (Sharma et al., 1975b; Childers, 1991), stimulate potassium conductance (North et al., 1987; Christie and North, 1988), and inhibit calcium conductance (Hescheler et al., 1987; Rhim and Miller, 1994). Opiates such as morphine bind to μ opioid receptors, which are distributed throughout brain regions that mediate reinforcement, analgesia, thermoregulation, and cardiopulmonary function (Herkenham and Pert, 1982). Chronic opiate administration leads to tolerance and dependence, and opiate withdrawal symptoms include irritability, insomnia, anorexia, gastrointestinal disturbances, chills, sweating, and increased heart rate and blood pressure (Way et al., 1969; Wei et al., 1973; Jaffe, 1990).

Numerous studies have examined neuronal mechanisms that may underlie opiate tolerance and dependence. Chronic opioid treatment of cultured cell lines results in receptor downregulation and desensitization (Law et al., 1983; Puttfarcken et al., 1988) and compensatory increases in adenylyl cyclase activity (Sharma et al., 1975a; Yu et al., 1990). Studies of chronic opiate administration in animals generally reveal no change in opioid receptor number (Klee and Streaty, 1974; Hollt et al., 1975; Childers et al., 1977) or mRNA levels (Brodsky et al., 1995), although decreased (Davis et al., 1979; Tao et al., 1987) or increased (Brady et al., 1989) opioid receptor density has been reported. Thus, the neuronal basis of opiate tolerance and dependence may involve postreceptor events such as receptor desensitization (Werling et al., 1986; Tao et al., 1993). G-protein involvement is further indicated by increased levels of $G_{i/o}\alpha$ in the locus coeruleus (LC) (Nestler et al., 1989) and amygdala (Terwilliger et al., 1991) and decreased $G_i\alpha$ in the nucleus accumbens (Terwilliger et al., 1991) after chronic morphine administration. Moreover, increased adenylyl cyclase, protein kinase A, and phosphoproteins have been reported in brain after chronic morphine treatment (Nestler et al., 1994).

The implications of altered G-protein levels in opiate tolerance and dependence are somewhat limited, because changes in protein or mRNA levels do not necessarily reflect alterations in functional signal transduction. Recently, a technique has been developed in which the binding of [^{35}S]GTPγS, in the presence of excess GDP, is used to assay receptor-activated G-proteins in isolated membranes (Hilf et al., 1989; Lorenzen et al., 1993; Sim et al., 1995; Traynor and Nahorski, 1995). When this technique was used, decreased basal and μ opioid agonist-stimulated [^{35}S]GTPγS binding were found in LC membranes from rats treated with chronic morphine (D. Selley, E. Nestler, and S. Childers, unpublished observations). Recently, our laboratory has developed an anatomical method, based on the [^{35}S]GTPγS membrane binding assay, that uses [^{35}S]GTPγS autoradiography to identify receptor-activated G-proteins in brain sections (Sim et al., 1995). This technique demonstrates specific receptor-activated G-proteins with high anatomical resolution. To determine whether chronic morphine treatment produces regional alter-

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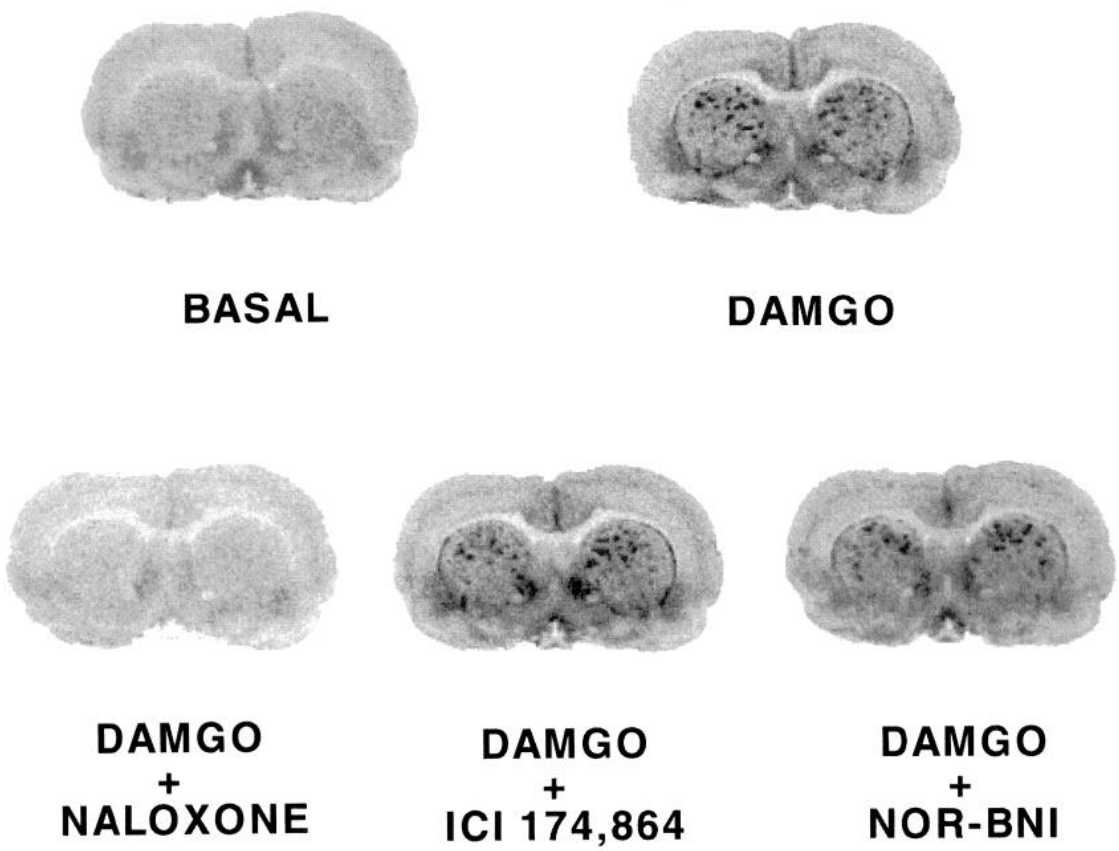

Figure 1. DAMGO-stimulated [35S]GTPγS binding in the forebrain in the presence of various opioid antagonists. Sections were incubated with 2 mM GDP and then with [35S]GTPγS (0.04 nM), 2 mM GDP, and 1 μM DAMGO with and without 0.1 μM naloxone, 1 μM ICI 174,864, or 0.1 μM nor-BNI. Basal binding was assessed in the absence of agonist.

ations in μ opioid receptor-coupled G-protein activity, the present study was performed to visualize μ opioid agonist-stimulated [35S]GTPγS binding in the rat brain after acute and chronic morphine administration.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (200–250 gm) were purchased from Zivic-Miller (Zelienople, PA). [35S]GTPγS (1150–1395 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and GDP were obtained from Sigma Chemical (St. Louis, MO). ICI 174,864 and nor-binaltorphimine (nor-BNI) were purchased from Research Biochemicals International (Natick, MA). GTPγS was purchased from Boehringer Mannheim (Indianapolis, IN). Reflections autoradiography film was purchased from New England Nuclear. All other reagent grade chemicals were obtained from Sigma Chemical or Fisher Scientific (Orangeburg, NY).

Morphine treatment. Acute and chronic morphine administration were performed according to previously published protocols (Kluttz et al., 1995). For acute morphine treatment, rats were injected intraperitoneally with either saline or 20 mg/kg morphine. After 1 hr, animals were killed by decapitation, and brains were processed as described below. For chronic morphine administration, rats were surgically implanted with a jugular vein catheter. After a 4–5 d recovery period, animals received either saline or 10 $mg \cdot kg^{-1} \cdot d^{-1}$ morphine (0.20 ml of 0.70 mg/ml morphine sulfate delivered hourly via the catheter). This dose was doubled every other day for morphine-treated animals, until the dose reached 320 $mg \cdot kg^{-1} \cdot d^{-1}$. After this 12 d treatment period, both saline- and morphine-treated animals were killed by decapitation.

In vitro [35S]GTPγS autoradiography. [35S]GTPγS autoradiography was performed as described previously (Sim et al., 1995). Animals were killed by decapitation, and the brains were removed and immediately immersed in isopentane at −35°C. Twenty micron coronal sections of appropriate regions were cut on a cryostat and thaw-mounted onto gelatin-subbed slides. Sections were processed by rinsing in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) at 25°C and then incubating with 2 mM GDP in assay buffer for 15 min at 25°C. Sections were then incubated for 2 hr at 25°C in assay buffer with [35S]GTPγS (0.04 nM) and 2 mM GDP, with and without appropriate agonists and antagonists. After incubation, slides were rinsed twice in cold 50 mM Tris-HCl buffer, pH 7.4, and once in deionized water, dried well, and exposed to film for 48–96 hr. Films were digitized with a Sony XC-77 video camera and analyzed using the National Institutes of Health Image program for Macintosh computers. Images were quantitated by densitometric analysis with [14C] standards. Values are expressed as nanocurie/gram of tissue and corrected for [35S] on the basis of incorporation of [35S] into sections of frozen brain paste. Radioactivity in each section was determined by liquid scintillation spectrophotometry, and sections were weighed to obtain nanocurie/gram of tissue for [35S]. [14C] standards and [35S] sections were then exposed to film and analyzed densitometrically, and correction factors were calculated to convert [14C] values to [35S] data. Data are reported as mean values ± SE of triplicate sections of brains from at least five animals. Statistical significance of the data was determined by the nonpaired two-tailed Student's *t* test using JMP (SAS Institute, Cary, NC).

RESULTS

In vitro autoradiography of DAMGO-stimulated [35S]GTPγS binding

[35S]GTPγS autoradiography was used to identify μ opioid receptor activation of G-proteins by measuring DAMGO-stimulated [35S]GTPγS binding. To verify the μ receptor specificity of DAMGO-stimulated [35S]GTPγS binding, sections were incubated with DAMGO in the presence and absence of μ-(naloxone), δ-(ICI-174,864), or κ-(nor-BNI) selective antagonists. Concentrations of antagonists were chosen so that >90% of the appropriate agonist-stimulated [35S]GTPγS binding was in-

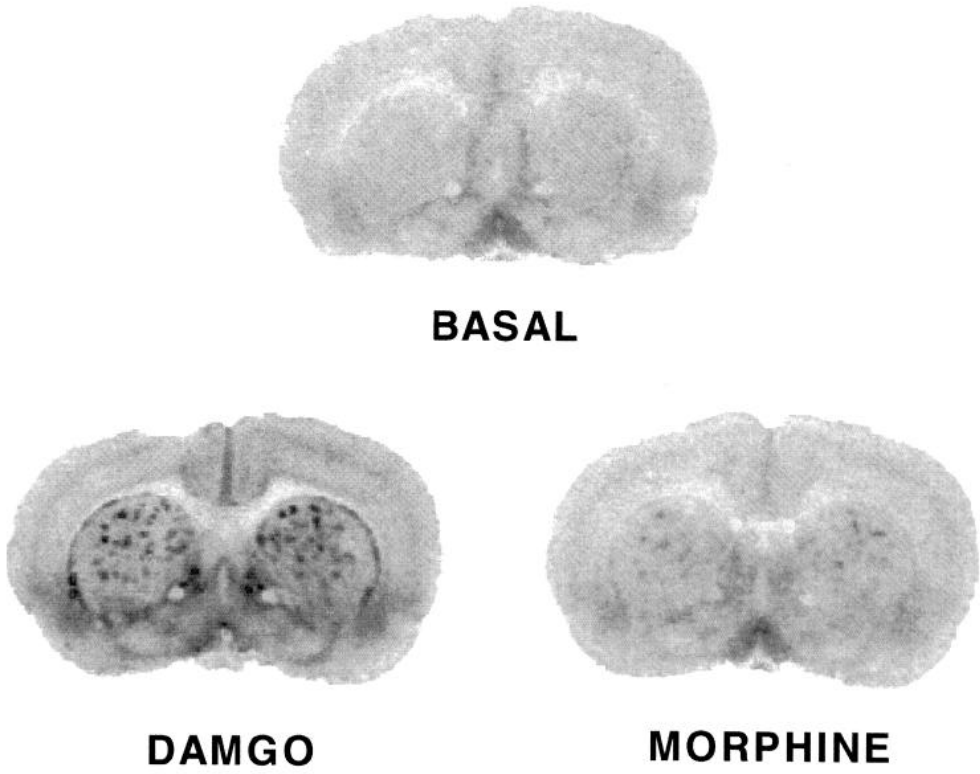

Figure 2. Comparison of [35S]GTPγS binding stimulated by DAMGO and morphine. Sections were incubated with 2 mM GDP and then with [35S]GTPγS (0.04 nM) and 2 mM GDP in the presence and absence of 3 μM DAMGO or 10 μM morphine. Basal binding was assessed in the absence of agonist.

hibited by the antagonist (data not shown). As shown in Figure 1, naloxone completely blocked DAMGO-stimulated [35S]GTPγS binding, so that the level of [35S]GTPγS binding was comparable to basal levels; however, incubation with either ICI-174,864 or nor-BNI had no effect on DAMGO-stimulated [35S]GTPγS binding, thus confirming the μ selectivity of this agonist.

The present study was designed to examine the effect of chronic morphine treatment on DAMGO-stimulated [35S]GTPγS binding; however, Traynor and Nahorski (1995) reported that morphine is a partial agonist in stimulating [35S]GTPγS binding to μ receptors in SH-SY5Y cell membranes. To determine whether morphine is also a partial agonist in brain, DAMGO-stimulated [35S]GTPγS binding was compared with that of morphine. Agonist concentration effect curves in brain membranes (data not shown) produced results similar to those obtained in SH-SY5Y cell membranes: maximally effective concentrations of morphine (5–10 μM) stimulated [35S]GTPγS binding by <60% of the magnitude observed with maximally effective concentrations of DAMGO. Similar results were also observed in brain sections (Fig. 2), where the level of [35S]GTPγS binding stimulated by DAMGO was greater than that stimulated by morphine, reflecting the greater efficacy of DAMGO versus morphine for G-protein activation (Traynor and Nahorski, 1995). Nevertheless, the distribution of labeling stimulated by both agonists, particularly in the patches of the caudate-putamen, was consistent with the finding that both morphine and DAMGO are agonists at μ receptors.

A regional analysis of DAMGO-stimulated [35S]GTPγS binding was performed by measuring the absolute levels of basal and DAMGO-stimulated [35S]GTPγS binding in control animals (Fig. 3). These results showed that similar basal levels of [35S]GTPγS binding (measured in the absence of agonist) were found throughout the brain, with the exception of the commissural nucleus tractus solitarius (cNTS), hypothalamus, and amygdala, which had elevated levels of basal [35S]GTPγS binding relative to other regions. DAMGO-stimulated [35S]GTPγS binding was relatively high in regions previously reported to contain high levels of μ opioid receptors (Herkenham and Pert, 1982), with the highest levels of DAMGO-stimulated [35S]GTPγS binding found in the telencephalon and diencephalon. No significant DAMGO-stimulated [35S]GTPγS binding was detected in the cerebellum, in agreement with the known distribution of μ opioid receptors in rat brain (Herkenham and Pert, 1982).

Effects of morphine administration on DAMGO-stimulated [35S]GTPγS autoradiography

[35S]GTPγS autoradiography was performed on brain sections at several levels from both acute and chronic morphine-treated rats and from the appropriate saline controls to determine whether morphine administration alters μ opioid receptor activation of G-proteins. Areas of significant DAMGO-stimulated [35S]GTPγS binding were measured, as shown in Figure 3. Data from Figure 3 and similar data from morphine-treated rats were used to calculate results from acute and chronic morphine-treated and control rats (Tables 1 and 2), where values are expressed as percentage of control basal binding in each region. In both tables, values are divided according to the level of the section (forebrain, diencephalon, or brainstem). Table 1 shows the effect of acute morphine treatment (20 mg/kg) on basal and DAMGO-stimulated [35S]GTPγS binding. These results showed that acute morphine treatment had no effect on basal levels of binding. The amount of DAMGO-stimulated binding varied considerably among regions, from 130% stimulation in the LC to 240% stimulation in the nucleus accumbens. Acute morphine treatment, however, had no effect on DAMGO-stimulated [35S]GTPγS binding in any of the regions examined.

The effects of chronic morphine treatment on DAMGO-stimulated [35S]GTPγS binding are shown in Table 2. In several forebrain areas (cingulate cortex, nucleus accumbens, and

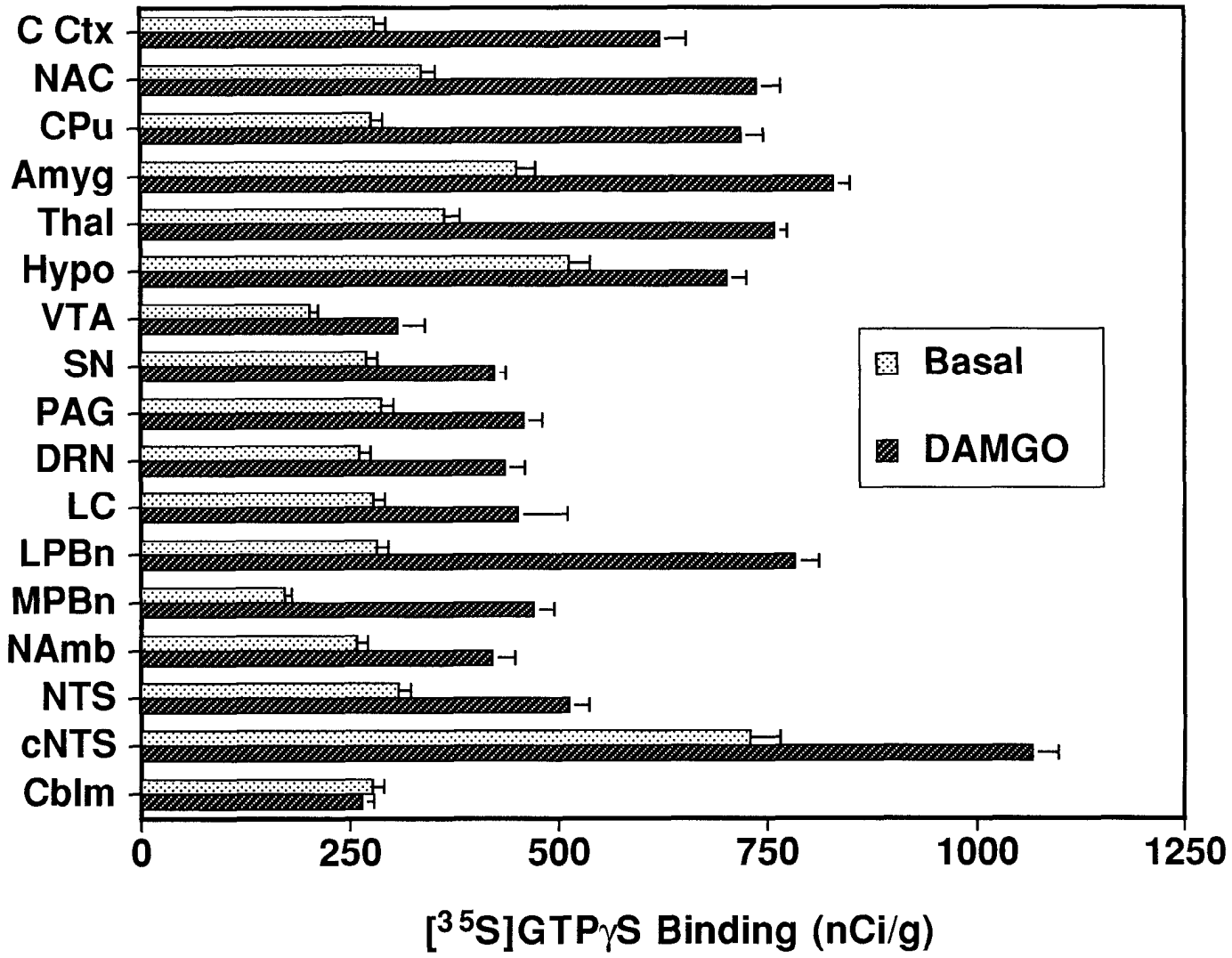

Figure 3. Regional comparison of basal and DAMGO-stimulated [^{35}S]GTPγS binding in brain sections from control rats. Sections were incubated with 2 mM GDP and then with [^{35}S]GTPγS (0.04 nM) and 2 mM GDP in the presence and absence of 10 μM DAMGO. [^{35}S]GTPγS binding is expressed as mean nanocurie/gram ± SE from triplicate sections of eight animals. *C Ctx*, Cingulate cortex; *NAC*, nucleus accumbens; *CPu*, caudate putamen; *Amyg*, amygdala; *Thal*, thalamus; *Hypo*, hypothalamus; *VTA*, ventral tegmental area; *SN*, substantia nigra; *PAG*, periaqueductal gray; *DRN*, dorsal raphe nucleus; *LC*, locus coeruleus; *LPBn*, lateral parabrachial nucleus; *MPBn*, medial parabrachial nucleus; *NAmb*, nucleus ambiguus; *NTS*, nucleus tractus solitarius; *cNTS*, commissural nucleus tractus solitarius; *Cblm*, cerebellum.

caudate-putamen), DAMGO stimulation of [^{35}S]GTPγS binding was relatively high (~200% stimulation compared with basal); however, neither DAMGO-stimulated nor basal levels of [^{35}S]GTPγS binding in the forebrain were affected by chronic morphine treatment (Fig. 4*A*). Similar results were observed in several areas at the level of the diencephalon, including the amygdala, thalamus, and hypothalamus (Table 2), where DAMGO-stimulated [^{35}S]GTPγS binding ranged from moderate to high. Once again, chronic morphine treatment had no significant effect on either basal or DAMGO-stimulated [^{35}S]GTPγS binding in these regions.

Interestingly, the only brain regions in which basal or DAMGO-stimulated [^{35}S]GTPγS binding differed between sections from control and chronic morphine-treated animals were found consistently in the brainstem. In these studies, the brainstem was sectioned at six different levels: 1) the substantia nigra and caudal ventral tegmental area (VTA); 2) the periaqueductal gray (PAG) and dorsal raphe nucleus (DRN); 3) the parabrachial nucleus (PBn); 4) the LC, at a level slightly caudal to the PBn; 5) the rostral medulla, including the NTS and nucleus ambiguus; and 6) the caudal medulla at the level of the cNTS. Results (Table 2) showed that in all of these brainstem nuclei, DAMGO-stimulated [^{35}S]GTPγS binding exhibited a wide range of activation, from relatively low (135% stimulation in the VTA) to high (230% stimulation in the PBn).

In brainstem sections that included the substantia nigra and caudal VTA, chronic morphine treatment had no significant effect on basal or DAMGO-stimulated [^{35}S]GTPγS binding. In the DRN, chronic morphine treatment produced a significant decrease in DAMGO-stimulated [^{35}S]GTPγS binding, with no significant change in basal [^{35}S]GTPγS binding. In the PAG, a nonsignificant downward trend in DAMGO-stimulated [^{35}S]GTPγS binding was observed after chronic morphine treatment. Although the anatomy and function of the dorsal and ventral PAG differ, similar results were obtained when this area was divided into dorsal and ventral regions or measured as a

Table 1. Effect of acute morphine treatment on basal and DAMGO-stimulated [^{35}S]GTPγS binding in the rat brain

	Basal		DAMGO-stimulated	
Region	Control	Acute morphine	Control	Acute morphine
Forebrain				
Nucleus accumbens	100 ± 7%	112 ± 9%	240 ± 15%	239 ± 11%
Caudate-putamen	100 ± 4%	114 ± 8%	216 ± 18%	228 ± 9%
Diencephalon				
Amygdala	100 ± 4%	96 ± 5%	151 ± 8%	157 ± 4%
Thalamus	100 ± 4%	95 ± 4%	181 ± 9%	193 ± 3%
Hypothalamus	100 ± 4%	93 ± 4%	121 ± 3%	132 ± 4%
Brainstem				
Substantia nigra	100 ± 9%	89 ± 5%	138 ± 12%	148 ± 5%
PAG	100 ± 5%	103 ± 7%	158 ± 6%	168 ± 9%
Dorsal raphe nucleus	100 ± 3%	99 ± 6%	150 ± 4%	161 ± 8%
Locus coeruleus	100 ± 4%	107 ± 15%	130 ± 6%	135 ± 16%
Lateral parabrachial nucleus	100 ± 10%	104 ± 8%	178 ± 16%	217 ± 16%
Medial parabrachial nucleus	100 ± 6%	106 ± 8%	189 ± 9%	185 ± 13%

Sections were incubated with 2 mM GDP and then with [^{35}S]GTPγS (0.04 nM) and 2 mM GDP, in the presence and absence of 10 μM DAMGO. Data are expressed as percentage of control basal binding and represent mean values ± SE of triplicate sections from five animals.

whole. In the PBn, chronic morphine treatment produced significant decreases in DAMGO-stimulated [^{35}S]GTPγS binding in both the lateral and medial subdivisions of this nucleus (Fig. 4*B*). Chronic morphine administration also produced a small but significant decrease in basal [^{35}S]GTPγS binding in the lateral PBn (LPBn). In the LC, significant decreases were identified in both basal and DAMGO-stimulated [^{35}S]GTPγS binding in sections from chronic morphine-treated rats (Fig. 4*C*). In the medulla, no significant chronic morphine-induced changes were observed in either the NTS or nucleus ambiguus; however, chronic morphine treatment did produce a significant decrease in DAMGO-stimulated [^{35}S]GTPγS binding in the cNTS, at the caudal extent of the nucleus (Fig. 4*D*), whereas no significant change in basal [^{35}S]GTPγS binding was measured in the cNTS.

DISCUSSION

Decreases in μ opioid-stimulated [^{35}S]GTPγS binding were identified in several brainstem nuclei, including the DRN, PBn, LC, and cNTS, after chronic morphine treatment. These changes do not seem to be a nonspecific artifact of the opiate treatment, for several reasons. First, significant changes in DAMGO-stimulated [^{35}S]GTPγS binding in sections from chronic morphine-treated rats occurred in the same direction, i.e., a decrease in DAMGO-stimulated binding. Second, significant changes in sections from

Table 2. Effect of chronic morphine treatment on basal and DAMGO-stimulated [^{35}S]GTPγS binding in the rat brain

	Basal		DAMGO-Stimulated	
Region	Control	Chronic morphine	Control	Chronic morphine
Forebrain				
Cingulate cortex	100 ± 5%	96 ± 5%	191 ± 4%	182 ± 8%
Nucleus accumbens	100 ± 4%	96 ± 4%	193 ± 7%	191 ± 5%
Caudate-putamen	100 ± 4%	97 ± 3%	219 ± 7%	229 ± 6%
Diencephalon				
Amygdala	100 ± 4%	103 ± 4%	169 ± 4%	169 ± 4%
Thalamus	100 ± 4%	100 ± 2%	186 ± 3%	184 ± 3%
Hypothalamus	100 ± 4%	101 ± 4%	131 ± 4%	135 ± 5%
Brainstem				
Ventral tegmental area	100 ± 7%	102 ± 4%	135 ± 11%	136 ± 6%
Substantia nigra	100 ± 2%	100 ± 6%	142 ± 4%	143 ± 7%
PAG	100 ± 3%	105 ± 4%	144 ± 6%	134 ± 4%
Dorsal raphe nucleus	100 ± 4%	98 ± 5%	144 ± 2%	131 ± 2%**
Locus coeruleus	100 ± 8%	74 ± 4%*	146 ± 16%	98 ± 6%*
Lateral parabrachial nucleus	100 ± 3%	84 ± 6%*	232 ± 8%	161 ± 9%**
Medial parabrachial nucleus	100 ± 3%	99 ± 5%	211 ± 9%	177 ± 6%**
Nucleus ambiguus	100 ± 2%	97 ± 4%	146 ± 8%	136 ± 6%
NTS	100 ± 3%	102 ± 6%	150 ± 6%	155 ± 6%
cNTS	100 ± 2%	95 ± 4%	141 ± 4%	130 ± 3%*

Sections were incubated with 2 mM GDP and then with [^{35}S]GTPγS (0.04 nM) and 2 mM GDP, with and without 10 μM DAMGO. Data are expressed as percentage of control basal binding and represent mean values ± SE of triplicate sections from at least seven animals. (*$p < 0.05$; **$p < 0.01$.) NTS, Nucleus tractus solitarius; cNTS, commissural NTS.

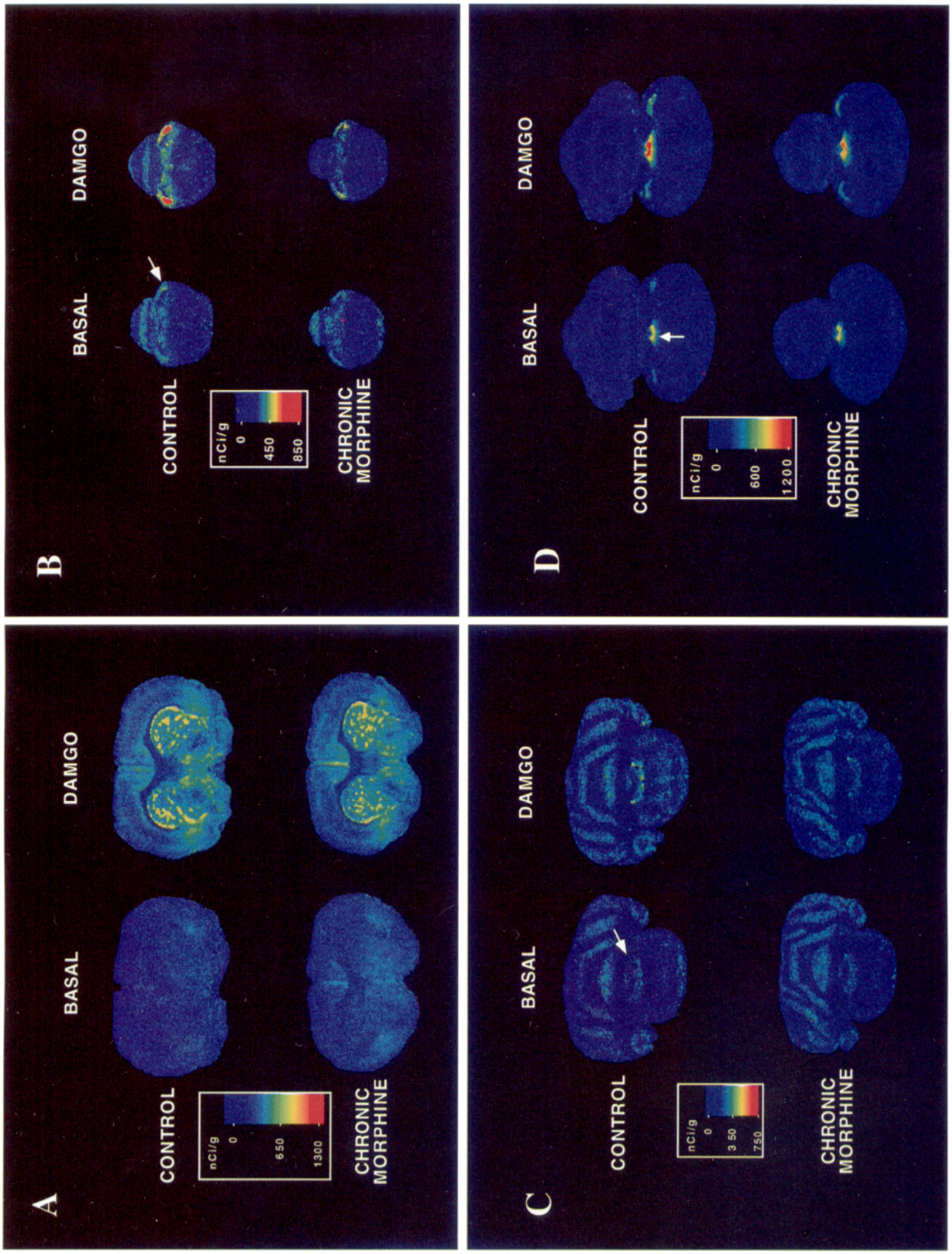

Figure 4. Brain sections comparing [35S]GTPγS autoradiography in control and chronic morphine-treated rats. Sections were incubated with 2 mM GDP and then with [35S]GTPγS (0.04 nM) and 2 mM GDP in the presence and absence of 10 μM DAMGO. Basal binding (assessed in the absence of DAMGO) is shown on the *left*, and agonist-stimulated [35S]GTPγS binding is shown on the *right*. Sections from control (*top*) and chronic morphine-treated (*bottom*) rats are shown at the level of the (*A*) caudate-putamen, (*B*) parabrachial nucleus (located bilaterally in the lateral pons), (*C*) LC (located bilaterally in the medial pons), and (*D*) commissural NTS (located in the dorsal medial medulla). Specific brainstem nuclei are indicated by *arrows*.

chronic morphine-treated animals were not distributed randomly across various brain regions but were localized to specific brainstem structures. It is also important to note that some negative findings in this study may be false negatives because some chronic morphine-induced changes may be too small to be detected autoradiographically. Because [^{35}S]GTPγS autoradiography detects overall G-protein activation, changes in the activity of one subtype of G-protein within the overall population may not be detected. This may be one reason why chronic morphine treatment decreased basal [^{35}S]GTPγS binding in only two regions (LC and LPBn), but it decreased DAMGO-stimulated [^{35}S]GTPγS binding in several other brainstem nuclei. It is also evident from this study that chronic morphine-induced changes are highly region-specific. Thus, it is possible that changes occur in subnuclei that were not specifically examined in this study. Finally, chronic morphine-induced decreases did not occur only in areas of highest or lowest levels of DAMGO-stimulated [^{35}S]GTPγS binding. Areas with relatively high (i.e., caudate-putamen) or low (i.e., hypothalamus) levels of stimulation exhibited no effect of chronic morphine treatment. Conversely, areas that showed decreased DAMGO-stimulated [^{35}S]GTPγS binding after chronic morphine treatment included those with both high (PBn) and low (LC) levels of stimulation.

It is also unlikely that the chronic morphine-induced reductions in DAMGO-stimulated [^{35}S]GTPγS binding were caused by residual morphine in the incubation mixture, because no significant changes in DAMGO-stimulated binding were observed after acute injection of a high dose (20 mg/kg) of morphine. Moreover, in areas showing significant reductions after chronic morphine treatment, these decreases were most often observed in the agonist-stimulated, as opposed to basal, binding levels. If residual morphine were present in the incubation mixture, basal binding levels should increase, and DAMGO-stimulated binding should remain unchanged. In areas that showed changes in basal [^{35}S]GTPγS binding (e.g., LC and LPBn), chronic morphine treatment produced decreased, not increased, binding.

Although it is unclear why these chronic morphine-induced changes were restricted to brainstem nuclei, several functional aspects of these regions are potentially relevant to opioid pharmacology. β-Endorphin is synthesized in the arcuate nucleus and cNTS (Gee et al., 1983; Bronstein et al., 1992), which have distinct projections. Arcuate neurons innervate telencephalic and diencephalic structures and primarily midline brainstem nuclei, whereas cNTS neurons innervate primarily lateral brainstem nuclei (Joseph and Michael, 1988; Sim and Joseph, 1991, 1994). With the exception of the DRN, changes in μ opioid-stimulated [^{35}S]GTPγS binding were identified in regions innervated by both the arcuate nucleus and cNTS, and within the cNTS. Perhaps the small cNTS population of opiocortin neurons is more responsive to the effects of chronic opiates and therefore develops compensatory responses more readily.

Another interesting possibility is that these brainstem nuclei are associated with opiate physical dependence. The results of this study correlate with changes in Fos immunorcactivity during opiate withdrawal (Stornetta et al., 1993). The brainstem regions exhibiting changes in μ opioid activation of G-proteins regulate nociception, sympathetic activity, and cardiopulmonary function and are important in physiological homeostasis. Opioids modulate respiration via the NTS, nucleus ambiguus, and PBn (Denavit-Saubie et al., 1978), and they affect cardiovascular function through mechanisms in the NTS (Bellet et al., 1980; Hassen et al., 1982; Petty and DeJong, 1982). Studies have also demonstrated the involvement of the PBn in cardiovascular regulation (Mraovitch et al., 1982; Chamberlain and Saper, 1992). Both nuclei receive cardiopulmonary visceral afferents (Davies and Kalia, 1981; Hayward and Felder, 1995) and have reciprocal connectivity with each other, as well as with autonomic centers in the brainstem and hypothalamus (Krukoff et al., 1993; Sim and Joseph, 1994). Decreased μ opioid-stimulated [^{35}S]GTPγS binding identified in the cNTS and PBn may indicate that compensatory changes are found in these nuclei because there is a narrow range of function in which homeostasis is maintained.

The identification of changes in μ opioid-stimulated [^{35}S]GTPγS binding in the LC correlates with results in isolated LC membranes, which demonstrated decreased basal and DAMGO-stimulated [^{35}S]GTPγS binding after chronic morphine treatment (D. Selley, E. Nestler, and S. Childers, unpublished observations). The LC displays biochemical changes in response to chronic morphine administration, including increased adenylyl cyclase and protein kinase A and changes in genetic expression (Nestler et al., 1994). The finding of increased $G_{i/o}$ content in the LC from chronic morphine-treated rats (Nestler et al., 1989), however, does not correlate with the decrease in activity noted in this study or in LC membranes. This discrepancy illustrates the fact that assays of levels of G-proteins by pertussis toxin labeling or immunoblots may not be representative of the set of G-proteins that are responsible for functional receptor coupling.

The importance of the LC (Maldonado and Koob, 1993) and of α_2 adrenergic receptors in this region (Aghajanian, 1978) in opiate physical dependence is well established. Changes in G-protein activity in the cNTS observed in the present study also indicate that alleviation of pressor responses by the α_2 agonist clonidine during opiate withdrawal (Buccafusco, 1983) may be attributable partially to actions in the A2 cell group, in which catecholamines affect blood pressure (Zandberg et al., 1979). Clonidine also alleviates some of the aversive stimulus effects of opiate withdrawal (Kosten, 1994). The LC may influence these effects through its connections with the forebrain, brainstem, and spinal cord (Jones, 1991). In addition to noradrenaline, chronic opiate administration also alters serotonin neurotransmission, and serotonin may be involved in opiate tolerance and dependence (Way et al., 1968; Spampinato et al., 1985), although these results are somewhat controversial (Cheney and Goldstein, 1971). Serotonin, or other neurotransmitters in the DRN, may contribute to thermoregulatory disturbances during opiate withdrawal, based on anatomical and electrophysiological evidence of connectivity with hypothalamic nuclei that are important in thermoregulation (Werner and Bienek, 1985; Sim and Joseph, 1993). Furthermore, serotonergic neurons in the DRN are known to influence sleep-wake cycles, which may contribute to sleep disturbances during opiate withdrawal (Jacobs and Fornal, 1991).

Several of these brainstem nuclei are components of an endogenous analgesic system. Opiate administration and stimulation of endogenous opioid release in the midbrain elicit analgesia (Mayer and Hayes, 1975; Yeung et al., 1977; Oliveras et al., 1979). The PAG modulates nociception via the well defined PAG-nucleus raphe magnus-dorsal horn pathway (Basbaum et al., 1977; Fields et al., 1977; Fields and Anderson, 1978; Basbaum and Fields, 1979). Furthermore, ascending projections from the DRN to the thalamus modulate nociception (Qiao and Dafny, 1988; Sim and Joseph, 1992). In the present study, decreased DAMGO-stimulated [^{35}S]GTPγS binding was observed in the DRN, with a downward trend in the PAG, of chronic morphine-treated ani-

mals. The LC, PBn, and NTS may also modulate nociception (Segal and Sandberg, 1977; Girardot et al., 1987; Morgan et al., 1989). It is possible that analgesic tolerance may result from changes in signal transduction in these brainstem nuclei, although it is not clear which nuclei and neurotransmitter systems are important in this regard.

One surprising result of this study is the lack of effect of chronic morphine treatment on μ opioid-stimulated [^{35}S]GTPγS binding in the telencephalon and diencephalon, particularly in regions implicated in the reinforcing effects of opiates. Previous studies have reported changes in the levels of $G_{i/o}\alpha$ in forebrain after chronic morphine treatment (Terwilliger et al., 1991), but it is not known which receptors are coupled to these G-proteins. Moreover, changes in G-protein levels may actually be secondary to the functional changes in receptor-coupled G-protein activity after chronic morphine treatment. In the present study, chronic morphine-induced changes in μ opioid-stimulated G-protein activity were identified in the DRN and LC, which provide serotonergic and noradrenergic innervation of the forebrain, respectively. Thus, changes in signal transduction in the LC and DRN could result in functional changes in the forebrain. Although noradrenergic or serotonergic systems may contribute to some of the psychological effects of opiates, dopamine is generally associated with reinforcement (Di Chiara and Imperato, 1988), and common neuronal mechanisms have been proposed for opiate- and cocaine-induced reinforcement (Terwilliger et al., 1991). Increases in D_1-stimulated adenylyl cyclase and in levels of $G_s\alpha$, in the absence of opioid receptor desensitization, have been identified in striatal cultures chronically treated with morphine (Van Vliet et al., 1993). Although the present results do not demonstrate changes in opioid receptor-coupled G-protein activity that would affect dopamine systems at either the level of cell bodies or terminals, opioid modulation of dopamine neurotransmission could occur via intermediate systems. For example, changes in μ opioid activation of G-proteins were found in the DRN, which has ascending serotonergic projections (Lorens and Guldberg, 1974; Sim and Joseph, 1993; Van Bockstaele et al., 1993) that regulate dopamine release in the nucleus accumbens, either through direct projections or indirectly via the VTA (Yoshimoto and McBride, 1992).

The demonstration of changes in μ opioid receptor-mediated G-protein activation in specific brainstem nuclei demonstrates an advantage of the [^{35}S]GTPγS autoradiographic technique, which allows anatomical examination of receptor-activated G-proteins. Because this technique can simultaneously detect several receptor types by using different agonists in adjacent sections, it will be possible to determine whether parallel changes occur with other opioid and nonopioid receptor types in the same chronic morphine-treated animals. Changes in opioid receptor-coupled G-protein activity, particularly in monoaminergic systems, in the DRN, LC, PBn, and cNTS, may provide an anatomical substrate for opiate tolerance and physical dependence.

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