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Chronic Morphine Up-Regulates Gα12 and Cytoskeletal Proteins in Chinese Hamster Ovary Cells Expressing the Cloned μ Opioid Receptor

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ABSTRACT

A growing body of literature indicates that chronic morphine exposure alters the expression and function of cytoskeletal proteins in addition to the well established interactions between μ opioid receptors and G proteins. In the present study, we hypothesized that chronic morphine alters the expression and functional effects of Gα12, a G protein that regulates downstream cytoskeletal proteins via its control of RhoA. Our results showed that chronic morphine treatment decreased the expression of Gαi2 (64%) and Gαi3 (60%), had no effect of Gαo, and increased Gα12 (66%) expression in Chinese hamster ovary (CHO) cells expressing the cloned human μ opioid receptors (hMOR-CHO cells) but not in cells expressing a mutant μ opioid receptor that do not develop morphine tolerance and dependence (T394A-CHO cells). Morphine treatment had no significant effect on PAR-1 thrombin receptor-activated G protein activity, as measured by thrombin-stimulated guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding. Chronic morphine treatment significantly enhanced thrombin-stimulated RhoA activity and thrombin-stimulated expression of α-actinin, a cytoskeletal anchoring protein, in hMOR-CHO cells. Proteomic analysis of two-dimensional gel spots prepared from hMOR-CHO cells showed that morphine treatment affected the expression of a number of proteins associated with morphological changes. Up-regulation of Gα12 and α-actinin by chronic morphine was also observed in mouse brain. Viewed collectively, these findings indicate, for the first time, that chronic morphine enhances the Gα12-associated signaling system, which is involved in regulating cellular morphology and growth, supporting other findings that chronic morphine may alter cellular morphology, in addition to cellular function.

Opioid μ receptors are coupled primarily to G proteins of the Gi/Go family and modulate the function of effector molecules, such as adenylate cyclase and protein kinases (Standifer and Pasternak, 1997; Bohn et al., 2000; Williams et al., 2001). Continual exposure of μ opioid receptor to μ agonists produces tolerance. The mechanisms underlying the development of opioid tolerance and dependence are complex and not fully elucidated. At the cellular level, chronic drug exposure leads to changes in receptor-effector signaling (Nestler and Aghajanian, 1997; Law et al., 2004; Waldhoer et al., 2004), including changes in receptor number and affinity, receptor uncoupling from transducer proteins, changes in effector molecule expression, increased activity by antiopioid peptides (Rothman, 1992; Rothman et al., 1993), and changes in G protein function and/or altered signaling proteins (Gintzler and Chakrabarti, 2000). Emerging data support the hypothesis that receptor desensitization, phosphorylation, and endocytosis are the underlying molecular mechanisms of physiological tolerance (Waldhoer et al., 2004). Importantly, other data indicate that particular G protein subunits and regulator of G protein signaling proteins (Connor and Christie, 1999; Garzon et al., 2001; Nakagawa et al., 2001; Zachariou et al., 2003; Xu et al., 2004) participate in modu-

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ABBREVIATIONS: hMOR-CHO, CHO cells expressing the cloned human μ opioid receptor; CHO, chinese hamster ovary; T394A-CHO, CHO cells expressing the cloned mutant μ opioid receptor; PBS, phosphate-buffered saline; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; SA, specific activity; SPA, scintillation proximity assay; RBD, Rho-binding domain; 2D, two-dimensional; PAR-1, protease-activated receptor-1.

lating opioid signaling, which may contribute to the development of opioid tolerance and dependence. More recent studies indicated that chronic morphine alters the expression and function of cytoskeletal proteins (Noble et al., 2000; Garcia-Sevilla et al., 2004; Marie-Claire et al., 2004), and chronic exposure to drugs of abuse produces persistent changes in the structure of dendrites and dendritic spines on cells in brain regions (Robinson and Kolb, 2004). Numerous studies document that the small GTPase RhoA is involved in the regulation of various cellular functions, such as remodeling of the actin cytoskeleton and induction of transcriptional activity. RhoA plays a central role in the organization of the cellular actin cytoskeleton through its ability to stimulate the formation of actomyosin-based structures and to regulate their contractility. Gα12/Gα13 are the major upstream regulators of RhoA activity (Vogt et al., 2003; Yamaguchi et al., 2003). Thus, signaling via the G protein Gα12 activates RhoA, which in turn regulates cellular growth and morphology.

In light of data showing that chronic morphine regulates cytoskeletal proteins, we hypothesized that chronic morphine exposure would alter the expression and functional effects of Gα12, a G protein that regulates downstream cytoskeletal proteins via its control of RhoA (Coughlin, 2000; Vogt et al., 2003). In the present study, we therefore investigated chronic morphine-induced changes in μ opioid receptor, various G protein α-subunits, and the Gα12-associated signaling system in cells expressing the cloned human μ opioid receptor (hMOR-CHO cells). We also assessed chronic morphine-induced G protein changes in cells expressing a mutant μ opioid receptor (T394A-CHO cells), which do not develop morphine tolerance and dependence (Deng et al., 2000; Xu et al., 2003), to determine whether any observed G protein changes are related to the development of opioid tolerance and dependence. The major findings of this study are that chronic morphine increases Gα12 expression (66%) in hMOR-CHO cells and mouse brain, but not in T394A-CHO cells, and that the increase in Gα12 expression is accompanied by activation of RhoA and Rho-dependent cytoskeletal responses.

Materials and Methods

Animals. Adult, male ICR mice (30–35 g; Harlan, Indianapolis, IN) were housed in groups of five in Plexiglas chambers with food and water available ad libitum. Animals were maintained in a temperature-controlled colony on a 12-h light/dark cycle. Studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Each mouse was implanted with a subcutaneous placebo or morphine (25 or 75 mg) pellet under brief ether anesthesia. The pellets remained in place for 72 h, at which time mice were sacrificed by cervical dislocation and the whole brains rapidly removed and frozen on dry ice. This procedure produces a high degree of opioid tolerance and dependence (Wang et al., 2001). After thawing brain tissue on ice, the appropriate central nervous system regions (cortex, caudate, and hippocampus) were dissected out using glass manipulators and homogenized by sonication in radioimmunoprecipitation assay buffer [1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 1 mM sodium orthovanadate in phosphate-buffered saline (PBS) buffer, pH 7.4]. Protein concentration was determined using the Pierce BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL). Homogenates were diluted and used for Western blot analysis. These brain regions were chosen because they are easily dissected and provide enough tissue for most biochemical analyses.

Cell Culture and Membrane Preparation. The recombinant CHO cells (hMOR-CHO or T394A-CHO) were produced by stable transfection with the human μ opioid receptor cDNA or mutant cDNA (Deng et al., 2000). The cells were grown on plastic flasks in 90% Ham's F-12 containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and G-418 (0.20–0.25 mg/ml) under 95% air/5% CO_2 at 37°C. To prepare membranes, cell pellets were suspended in 50 mM Tris-HCl, pH 7.4, containing 4 μg/ml leupeptin, 2 μg/ml chymostatin, 10 μg/ml bestatin, and 100 μg/ml bacitracin and homogenized using a polytron (Brinkmann Instruments, Westbury, NY) at setting 6 until a uniform suspension was achieved. The homogenate was centrifuged at 30,000*g* for 10 min at 4°C, and the supernatant was discarded. The membrane pellets were resuspended in binding buffer and used for receptor binding or [^{35}S]GTPγS binding assays. For morphine pretreatment experiments, the medium was changed, and then cells were incubated with morphine (1 μM) for 20 h. In some experiments, incubations proceeded in the absence or presence of 10 μM naloxone. Cells were washed three times with PBS, and cell membranes were prepared as described above. This treatment produces opioid tolerance to morphine (Xu et al., 2003).

Receptor Binding Assays. We used [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin ([^{3}H]DAMGO) (2.0 nM, SA = 45.5 Ci/mmol) to label μ binding sites. The K_d and B_{max} of μ receptors labeled by [^{3}H]DAMGO were determined by displacing two concentrations of [^{3}H]DAMGO (1.5 and 7.5 nM) each by eight concentrations of DAMGO (0.03815–2500 nM) as described in detail elsewhere (Xu et al., 2003). All assays took place in 50 mM Tris-HCl, pH 7.4, with a protease inhibitor cocktail (100 μg/ml bacitracin, 10 μg/ml bestatin, 4 μg/ml leupeptin, and 2 μg/ml chymostatin) in a final assay volume of 0.5 ml. Triplicate samples were filtered with Brandell Cell Harvesters (Biomedical Research and Development Inc., Gaithersburg, MD), over Whatman GF/B filters, after a 2- to 3-h incubation at 25°C. The nonspecific binding was determined using 20 μM levallorphan. The data obtained from three independent experiments were fit to the one-site binding model using the nonlinear least-squares curve fitting program MLAB-PC (Civilized Software, Bethesda, MD) for the best-fit estimates of the K_d and B_{max}.

Western Blotting of Various G Protein α-Subunits. For Western blot analysis of G protein α-subunits, cell monolayers were harvested and homogenized by sonication in radioimmunoprecipitation assay buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 1 mM sodium orthovanadate in PBS buffer, pH 7.4). Protein concentration was determined using the Pierce BCA Protein Assay Reagent Kit. Homogenates were diluted to a desired protein concentration with 2× SDS-polyacrylamide gel electrophoresis loading buffer (Invitrogen, Carlsbad, CA). Samples were boiled for 6 min and loaded into 8 to 16% polyacrylamide minigels (Invitrogen) for gel electrophoresis at 30 μg/lane. Proteins from gel are transferred to Immobilon-polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA) using a semidry apparatus (Bio-Rad, Hercules, CA). Nonspecific binding to membranes was prevented by blocking for 60 min at room temperature with PBS solution containing 5% nonfat dry milk. Membranes were then probed by overnight incubation (4°C) with 1:1000 dilution of rabbit polyclonal anti-G protein α-subunits antibodies (Calbiochem, San Diego, CA) or 1:2000 dilution of rabbit polyclonal antiopioid μ receptor antibody (Calbiochem) or 1:200 dilution of rabbit polyclonal anti-RhoA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were washed three times (10 min × 3 in Tris-buffered saline solution) and then incubated with a 1:5000 dilution of horseradish peroxidase conjugate secondary antibody in Tris-buffered saline solution, containing 0.5% nonfat dry milk for 90 min at room temperature. After washing three more times, antibody complex was visualized by chemiluminescence using a kit from Pierce Chemical. Western blots were digitized and

quantified using densitometric analysis (NIH Image software). Results from at least three experiments were analyzed using the program Prism (GraphPad Software Inc., San Diego, CA).

[^{35}S]GTPγS Binding by Antibody Capture and SPA Detection. We detected agonist-stimulated [^{35}S]GTPγS binding to particular G protein subunits using an antibody capture strategy, coupled to detection by scintillation proximity assay (SPA) as described (DeLapp et al., 1999). Briefly, hMOR-CHO cell membranes (10 μg) were incubated on 96-well Costar plates in the absence and presence of DAMGO (10 μM) and [^{35}S]GTPγS (0.2 nM) for 1 h at room temperature in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 3 μM GDP, and 5 mM $MgCl_2$ (total reaction volume is 200 μl). The reaction was stopped by solubilizing cell membranes with detergent (NP40, 0.3% final) and gentle agitation for 30 min. G protein α-subunit antibodies (Santa Cruz Biotechnology, Inc.) were then added (0.5 mg/well) and plates incubated for an additional 1 to 2 h to allow antibody-G_α complexes to form. At the end of the incubation period, SPA beads coated with anti-rabbit secondary antibody (Amersham Biosciences Inc., Piscataway, NJ) were added at the manufacturer's recommended concentration (50 μl/well), incubated for 3 h at room temperature, and then allowed to settle overnight (4°C) before counting on a Trilux liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Nonspecific binding was determined using 40 μM GTPγS. Data from several experiments were analyzed using the program Prism (GraphPad Software Inc.). Results are expressed as the mean ± S.E.M. (n = 5–6).

[^{35}S]GTPγS binding experiments designed to detect Gα12 activation by thrombin did not use the antibody capture method because of a low signal/noise ratio and were performed as described previously (Xu et al., 2003). Briefly, cell membranes (10 μg) were suspended in 500 μl of buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 50 μM GDP, 0.1% bovine serum albumin, 0.05 nM [^{35}S]GTPγS, and varying concentration of thrombin. The reaction was initiated by the addition of cell membranes and terminated after 1 h incubation at room temperature by the addition of 3 ml of cold (4°C) 10 mM Tris-HCl, pH 7.4, followed by rapid vacuum filtration through Whatman GF/B filters. The filters were then washed twice with 4 ml of cold 10 mM Tris-HCl, pH 7.4. Bound radioactivity was counted using a Trilux liquid scintillation counter at 60% efficiency. Nonspecific binding was determined in the presence of 40 μM GTPγS. Assays were performed in duplicate, and each experiment was performed four times. The EC_{50} (the concentration of thrombin that produces 50% maximal stimulation) and E_{max} (percentage of maximal stimulation) were determined using the program Prism (GraphPad Software Inc.).

RhoA Activity Assay. RhoA activity was assessed using the Rho-binding domain (RBD) of Rhotekin as described previously (Ren et al., 1999), and the assay procedures followed protocols provided by Upstate USA (Charlottesville, VA). To examine thrombin receptor-dependent RhoA activation, cells were stimulated with thrombin (1.02 units/ml) for 2 min. Cell lysates were incubated with Rhotekin RBD (Rhotekin Rho binding domain)-agarose slurry for 45 min at 4°C with gentle agitation and then washed three times. The RhoA content was determined by immunoblotting samples using rabbit anti-RhoA antibody (Santa Cruz Biotechnology, Inc.). Results from three experiments were analyzed using the program Prism (GraphPad Software Inc.).

Two-Dimensional Gel Electrophoresis and Liquid Chromatography-Tandem Mass Spectrometry Analysis of Protein Digests. hMOR-CHO cells were treated with morphine as described above. Briefly, 800 μl of osmotic lysis buffer (plus nucleases and protease inhibitors) and 400 μl of SDS boiling buffer without 2-mercaptoethanol were added to each cell pellet (control and morphine-treated). Cells were lysed, and protein determinations were done using the bicinchoninic acid assay. Each sample was then diluted to 1 mg/ml in SDS boiling buffer with 2-mercaptoethanol and placed in a boiling water bath for 5 min prior to loading. Two-dimensional electrophoresis and 2D protein gel staining were performed according to the method of O'Farrell (1975) by Kendrick Labs, Inc. (Madison, WI). Quantitative analysis of digitized images was carried out using Progenesis Discovery software (version 2003.03; Nonlinear Dynamics, Newcastle Upon Tyne, UK). Computerized comparison (duplicate gels averaged) identified 16 spots with altered protein level (≥2-fold) in the morphine-treated cells compared with control cells, among which seven spots were excised from the Coomassie blue-stained gels, reduced, and alkylated, followed by trypsinization as previously described (Boja et al., 2003). Proteolytic peptides were analyzed using a Micromass Q-TOF Ultima Global (Micromass, Manchester, UK) in electrospray mode interfaced with an Agilent HP1100 CapLC (Agilent Technologies, Palo Alto, CA) prior to the mass spectrometer. Eight microliters of each digest was loaded onto a Vydac C18 MS column (100 × 0.15 mm; Grace Vydec, Hesperia, CA), and chromatographic separation was performed at 1 μl/min using the following gradient: 0 to 10% B over 5 min, gradient from 10 to 40% B over 60 min, 40 to 95% B over 5 min, 95% B held over 5 min (solvent A, 0.2% formic acid in water; solvent B, 0.2% formic acid in acetonitrile). The top three most abundant ions observed in the preceding survey scan (m/z 300-1990) above a threshold of 10 counts/s were selected for collision-induced dissociation. Data were processed using the ProteinLynx to generate peak list files prior to in-house licensed Mascot search at http://biospec.nih.gov (Matrix-Science Ltd., London, UK).

Sources. [^{35}S]GTPγS (SA = 1250 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences. [^{3}H]DAMGO (SA = 45.5 Ci/mmol) and DAMGO were provided by Multiple Peptide System via the Research Technology Branch (National Institute on Drug Abuse). Catalog numbers are reported in parentheses. Thrombin was purchased from either Sigma-Aldrich (T9549) (St. Louis, MO) or Hematologic Technologies, Inc. (HCT-0020) (Essex Junction, VT). SPA beads coated with anti-rabbit secondary antibody were obtained from Amersham Biosciences Inc. (RPNQ0016) and Rhotekin RBD-agarose slurry from Upstate USA (14-383). For Western blots, antibodies directed against the μ opioid receptor (PC165L) and various G protein α-subunits were purchased from Calbiochem [Gα12 (371778), Gαo (371728), Gαi3 (371729), and Gαi2 (371727)]. The following antibodies were purchased from Santa Cruz Biotechnology, Inc.: Gαi3 (SC-262), for antibody capture assays and RhoA (SC-179), and α-actinin (SC-15335) for Westerns. Horseradish peroxidase-labeled secondary antibody was purchased from Amersham Biosciences Inc. (RPN1004). The sources of other agents are published (Xu et al., 2004).

Results

Effect of Morphine Treatment on the Expression of the μ Opioid Receptor and G Protein α-Subunits. We first determined the effect of chronic morphine treatment on the K_d and B_{max} of [^{3}H]DAMGO binding to cloned μ (wild-type) and mutant μ (mutant-type) receptors. As reported in Table 1,

TABLE 1

Saturation binding of [^{3}H]DAMGO to the cloned human μ or mutant μ opioid receptors

Two concentration of [^{3}H]DAMGO (1.5 and 7.5 nM) were each displaced by eight concentrations of DAMGO (0.03815 to 2500 nM). The data obtained from three independent experiments were fit to the one-site binding model using the nonlinear least squares curve fitting program MLAB-PC (Civilized Software) for the best-fit estimates of the K_d and B_{max}. The F-test was used to determine the corresponding P values (compared with control value) for the increase in the sum squares that resulted from the constraint. There were no significant differences between groups.

Condition	K_d	B_{max}
	nM ± S.E.M.	*fmol/mg protein*
hMOR-CHO cells		
Control	1.26 ± 0.16	1369 ± 121
Morphine-treated	1.69 ± 0.28	1381 ± 125
T394A-CHO cells		
Control	0.95 ± 0.26	1233 ± 121
Morphine-treated	1.42 ± 0.18	1469 ± 129

chronic morphine treatment did not change the K_d and B_{max} of [^{3}H]DAMGO binding in either hMOR-CHO or T394A-CHO cells. Western blot analysis confirmed no changes in the expression level of μ opioid receptor in control or morphine-treated hMOR-CHO and T394A-CHO cells (data not shown). As reported in Fig. 1A for hMOR-CHO cells, chronic morphine treatment decreased the expression of Gαi2 (64%) and Gαi3 (60%), had no effect of Gαo, and increased Gα12 (66%) expression. In contrast (Fig. 1B), chronic morphine treatment failed to alter the expression of these G protein α-subunits in T394A-CHO cells. The concurrent administration of a μ receptor antagonist, naloxone (10 μM), significantly blocked chronic morphine-induced up-regulation of Gα12 (Fig. 1C) and down-regulation of Gαi3 (Fig. 1D). These commercially available antibodies labeled bands with the appropriate molecular mass and in good agreement with previous studies (Fabian et al., 2002; Yamaguchi et al., 2003).

Effects of Morphine Treatment on Selective Coupling of Thrombin Receptor to Gα12 and Thrombin Receptor-Dependent RhoA Activation. Using antibody capture and SPA detection, we first determined basal and DAMGO-stimulated [^{35}S]GTPγS binding to Gαi-3 and Gα12 in hMOR-CHO cells. Our data (Fig. 2) showed that DAMGO-stimulated [^{35}S]GTPγS binding is mediated by Gαi3, since DAMGO significantly increased [^{35}S]GTPγS binding to Gαi3 from 100 (basal) to 170% (Fig. 2) and that DAMGO had no effect on stimulating [^{35}S]GTPγS binding to Gα12. Since the PAR-1 thrombin receptor signals via Gα12 in CHO cells, we further examined the effect of morphine treatment on selective coupling of the thrombin receptor to Gα12 and thrombin receptor-dependent RhoA activation. Using hMOR-CHO cell membranes (Fig. 3), we observed that thrombin stimulated [^{35}S]GTPγS binding in a dose-dependent manner. However, chronic morphine had no significant effect on the EC_{50} (3.93 $\pm$ 1.14 units/ml in control cells versus 4.87 $\pm$ 1.14 units/ml in morphine-treated cells) or E_{max} values (63 $\pm$ 2 in control cells versus 65 $\pm$ 3 in morphine-treated cells). Examination of downstream effects showed that chronic morphine substantially decreased basal RhoA activity (GTP-Rho), and thrombin activated RhoA activity in both control and morphine-treated hMOR-CHO cells and that the effect in morphine-

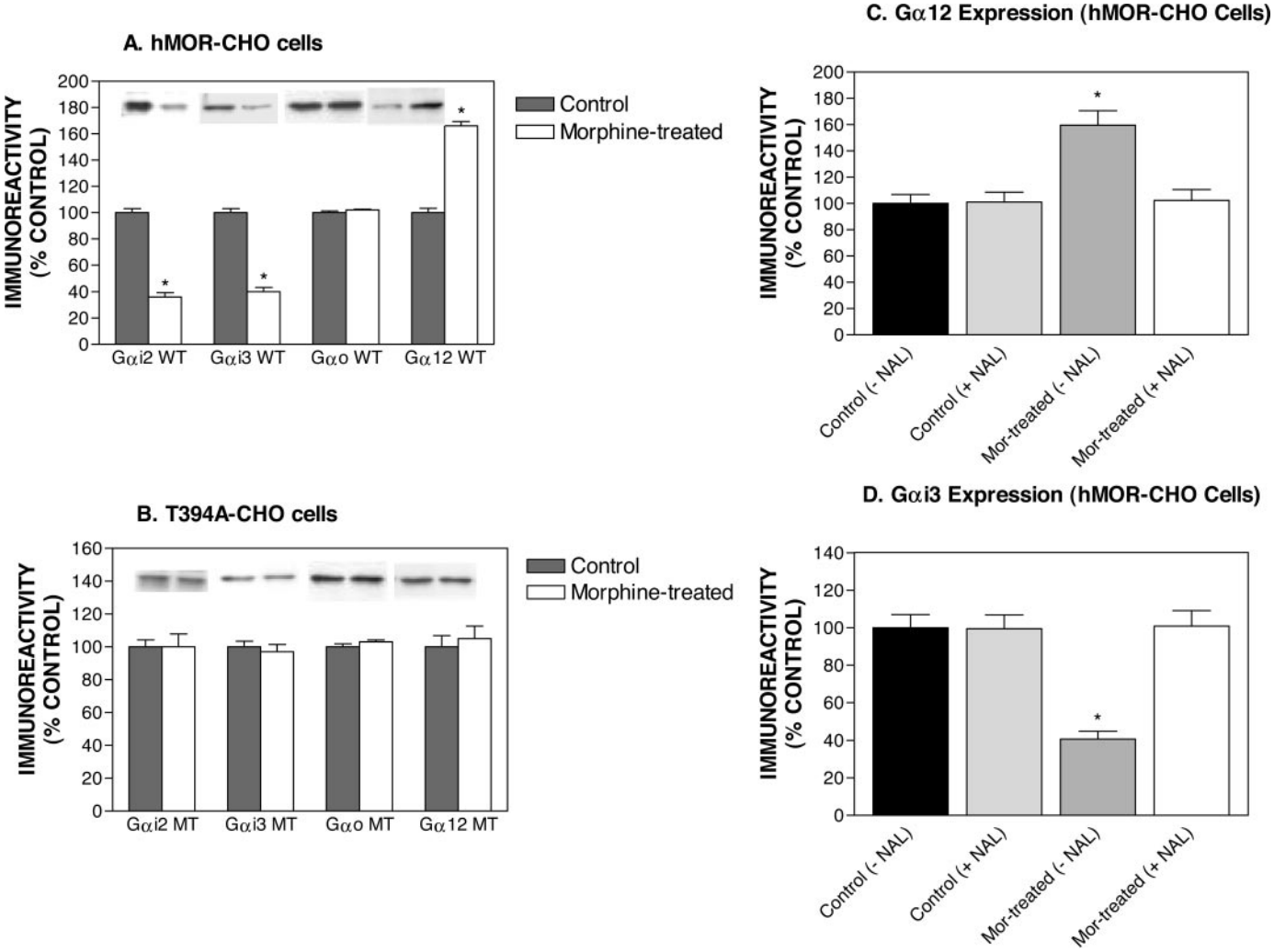

Fig. 1. A and B, comparison of Western blots of various G protein α-subunits by subtype-specific antibodies in the control or morphine-treated hMOR-CHO (A) and T394A-CHO (B) cells. Results are expressed as mean $\pm$ S.E.M. ($n = 3$). Representative Western blots are shown. *, $P < 0.05$ when compared with control cells (two-tailed Student's t test). C and D, comparison of the effects of naloxone (10 μM) on the expression level of Gα12 (C) and Gαi3 (D) in the control and morphine-treated hMOR-CHO cells. Results are expressed as mean $\pm$ S.E.M. ($n = 3$). *, $P < 0.05$ when compared with control cells (two-tailed Student's t test).

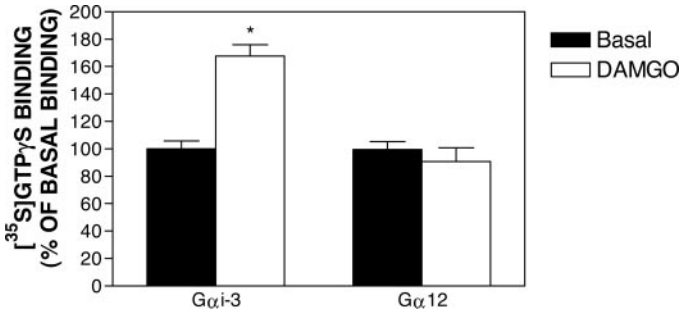

Fig. 2. Antibody capture assays for the basal and DAMGO-stimulated (10 μM) [^{35}S]GTPγS binding to Gαi3 and Gα12 protein in the control hMOR-CHO cells. Results are expressed as mean ± S.E.M. (n = 5–6). *, $P < 0.01$ when compared with basal binding (two-tailed Student's t test).

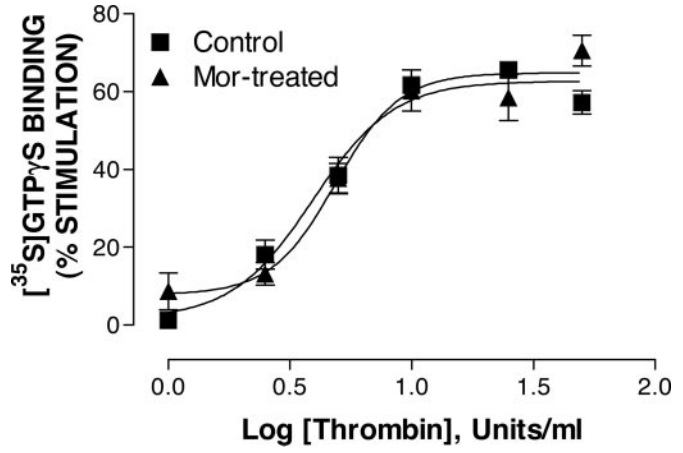

Fig. 3. Effects of morphine pretreatment on thrombin-stimulated [^{35}S]GTPγS binding in hMOR-CHO cell membranes. Results are expressed as mean ± S.E.M. (n = 4).

treated cells was greater (29% increase in control cells versus 140% increase in treated-cells) (Fig. 4A). In contrast, chronic morphine did not alter basal RhoA activity in T394A-CHO cells (Fig. 5) and did not change total Rho expression in either hMOR-CHO or T394A-CHO cells (data not shown). Importantly, thrombin stimulated the expression of α-actinin (a cytoskeletal anchoring protein with molecular mass of 97 kDa) (Lum and Malik, 1996) in morphine-treated cells but not in the control cells (Fig. 4B).

To determine whether similar changes occur in vivo, we implanted mice with placebo- or morphine-containing pellets according to a well established protocol that produces a high degree of morphine tolerance and dependence. We used two doses of pellets to test for a dose-response relationship. The results demonstrated that chronic morphine increased Gα12 expression in the caudate and hippocampus by 44 to 60% (Fig. 6A) and increased α-actinin expression by about 40% in the caudate but not in the hippocampus (Fig. 6B).

Proteomic Analysis of 2D Spots Altered by Chronic Morphine Treatment of hMOR-CHO Cells. Comparison (duplicate gels averaged) of polypeptide spots identified 16 spots with altered protein level (≥2-fold) in the morphine-treated cells. As reported in the Table 2, spots 48, 80, 91, 233, and 245 had increased protein expression, whereas spots 342 and 632 displayed decreased protein expression. Further protein identification by tandem mass spectrometry from 2D spots showed that morphine treatment down-regulated expression of sialidase 2 (*N*-acetyl-α-neuraminidase) and ribosomal protein and up-regulated expression of junction plakoglobin, phosphoglucomutase 1, heat shock protein cognate 71, ubiquilin, and tubulin β chain protein.

Discussion

Current work in many laboratories, including our own, is directed toward understanding opioid receptor signaling, regulation, and trafficking. We previously showed that chronic morphine results in functional uncoupling of the μ

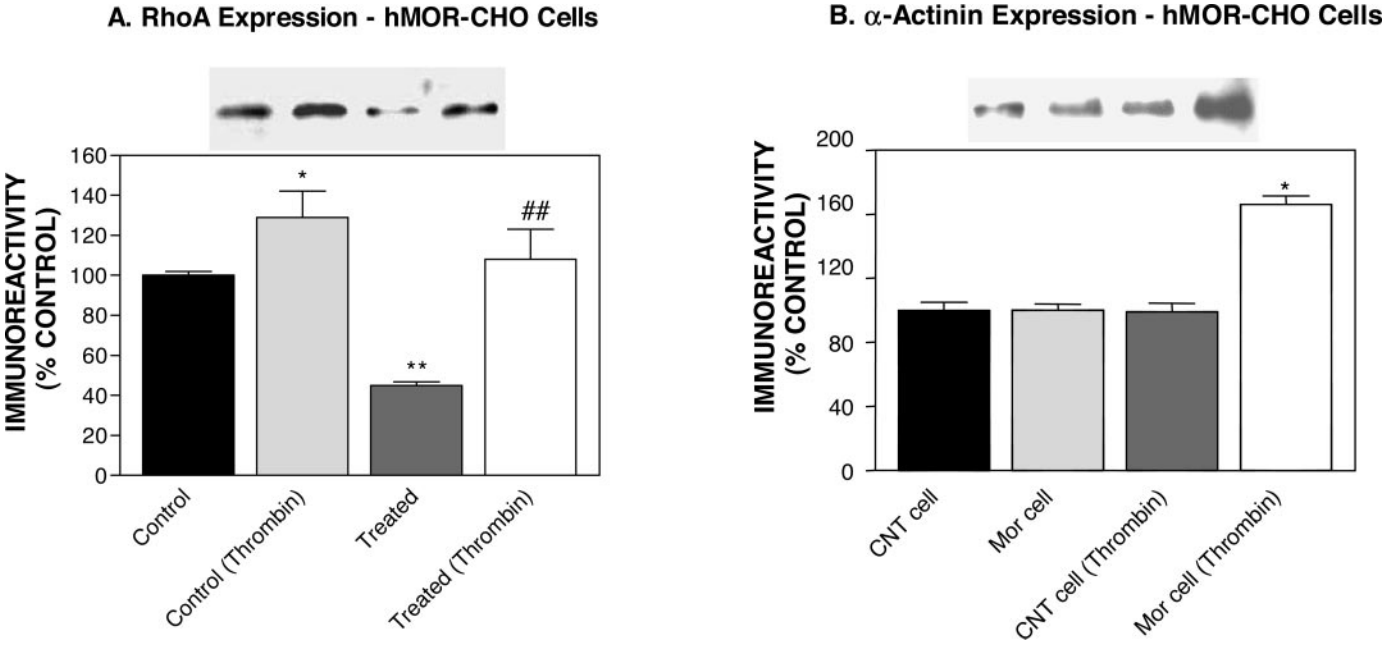

Fig. 4. Comparison of the effects of thrombin (1.02 units/ml, 2 min) on the expression of active RhoA (GTP-Rho) (A) and α-actinin protein (B) in the control and morphine-treated hMOR-CHO cells. Results are expressed as mean ± S.E.M. (n = 3). Representative Western blots are shown. *, $P < 0.05$; **, $P < 0.01$ when compared with control cells; ##, $P < 0.01$ when compared with morphine-treated cells (two-tailed Student's t test).

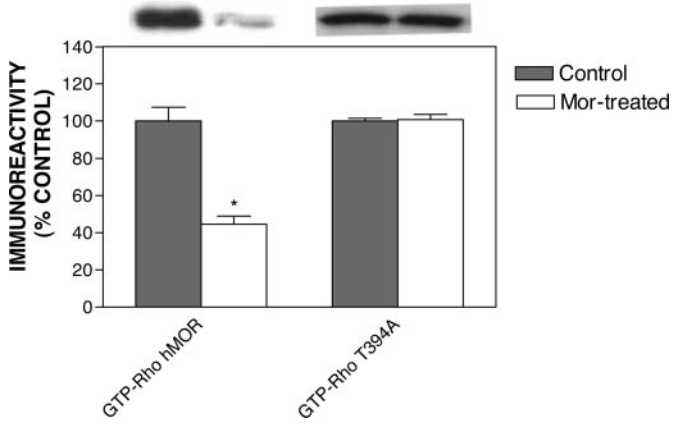

Fig. 5. Comparison of the effects of chronic morphine treatment on the expression of active RhoA (GTP-Rho) in hMOR-CHO and T394A-CHO cells. Results are expressed as mean ± S.E.M. (n = 3). Representative Western blots are shown. *, $P < 0.05$ when compared with control cells (two-tailed Student's t test).

opioid receptor from its G protein in CHO cells expressing the cloned human μ opioid receptors and in brain tissue prepared from rats rendered tolerant to morphine by implantation of morphine pellets (Xu et al., 2003). We observed that chronic morphine treatment produces a loss of the ability of μ opioid agonist to increase the B_{max} of the high-affinity [^{35}S]GTPγS binding sites, an increased EC_{50} for μ agonist-mediated inhibition of forskolin-stimulated cAMP accumulation, and an increase in the basal B_{max} of the high-affinity [^{35}S]GTPγS binding sites.

Mutagenesis analysis of several G protein-coupled receptors indicated that third cytoplasmic loops and the C terminus are most likely involved in both the coupling of receptors and G protein complexes and in the functional regulation of the receptors. Phosphorylation of serine or threonine residues located in the C terminus of the receptor is often implicated in receptor desensitization (Gainetdinov et al., 2004). Consistent with this general formulation, the C-terminally truncated μ opioid receptor and mutant receptor T394A both showed complete loss of DAMGO-induced desensitization (Deng et al., 2000). Our studies with the T394A-CHO cells confirmed that this mutation blocked the cellular changes associated with the development of morphine tolerance and dependence (Xu et al., 2003). As such, these cells are particularly useful to determine whether a chronic morphine-induced change is related to the development of tolerance and dependence.

Therefore, to further investigate the role of specific G protein α-subunits in morphine tolerance and dependence and to test our hypothesis that chronic morphine will increase expression of Gα12, we determined the effect of chronic morphine on the expression of the μ opioid receptor and particular G protein α-subunits in hMOR-CHO and T394A-CHO cells (Fig. 1). As reported by others (Terwilliger et al., 1991) in rat nucleus accumbens, we also observed a significant down-regulation of Gαi2/Gαi3 in morphine-treated hMOR-CHO cells but not in morphine-treated T394A-CHO cells. These results suggest that G protein subunits, such as Gαi-3 and Gαi-2, may contribute to morphine tolerance.

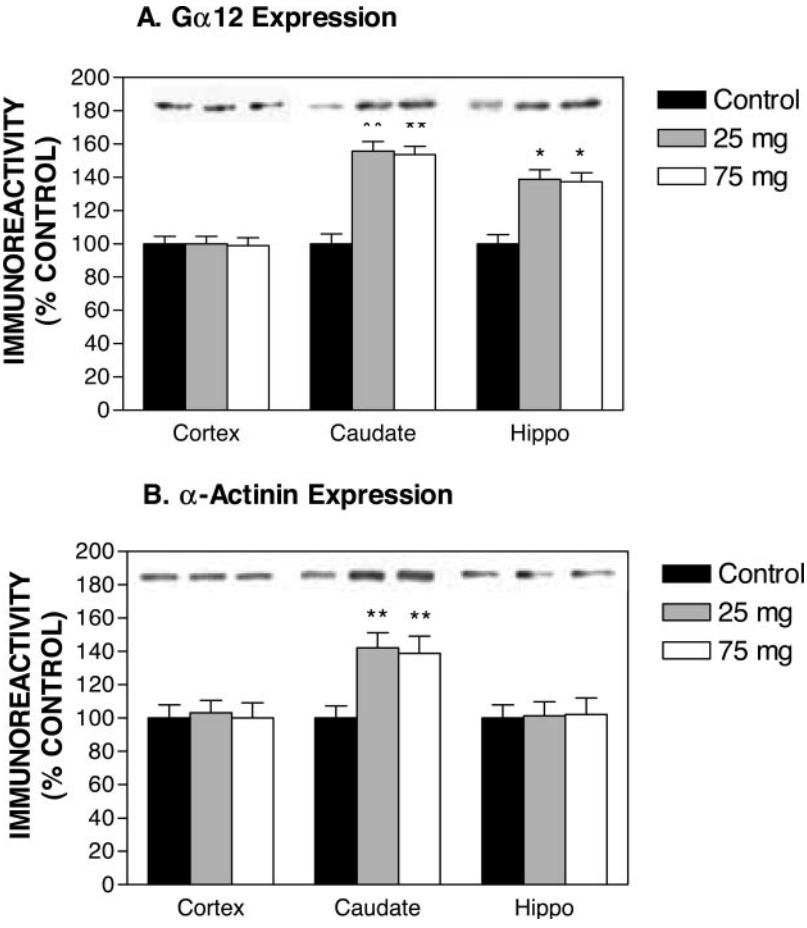

Fig. 6. Effects of chronic morphine treatment in the expression level of Gα12 (A) and α-actinin protein (B) in mice cortex, caudate, and hippocampus. Results are expressed as mean ± S.E.M. (n = 3). Representative Western blots are shown. *, $P < 0.05$; **, $P < 0.01$ when compared with control (two-tailed Student's t test).

TABLE 2

Protein expression altered by chronic morphine in hMOR-CHO cells

Samples were proteolyzed and analyzed using a Micromass Q-TOF Ultima Global as described under *Materials and Methods*. Data were processed using ProteinLinx to generate peak list files prior to an in-house licensed Mascot search at http://biospec.nih.gov (MatrixScience Ltd.).

Spot No.	Molecular Mass	pI	Protein Identified	Sequences from Database	Up- or Down-Regulation
	kDa				
48	86	6.33	Junction plakoglobin (desmoplakin III)	^{427}TLVTQNSGVEALIHAILR^{444} ^{583}LNTIPLFVQLLYSSVENIQR^{602}	Up Up
80	77	6.35	Phosphoglucomutase 1	^{108}GVVISFDAR^{116} ^{309}GVLTLSFALADKIK^{322} ^{5017}LFGLNR^{506} ^{521}FEISAIR^{527} ^{528}DLTTGYDDSQPDKK^{541}	
			Heat shock protein cognate 71	^{37}TTPSYVAFTDTER^{49} ^{50}LIGDAAK^{56} ^{160}DAGTIAGLNVLR^{171} ^{172}IINEPTAAAIAYGLDKK^{188} ^{329}SQIHDIVLVGGSTR^{342}	Up
91	74	5.26	Ubiquilin	^{222}NPEISHMLNNPDIMR^{236} ^{237}QTLELAR^{243}	Up
233	52	6.77	Tubulin β chain	^{63}AILVDLEPGTMDSVR^{77} ^{77}SGPFGQIFRPDNFVFGQSGAGNNWAK^{103} ^{104}GHYTEGAELVDSVLDVVR^{121} ^{155}IREEYPDR^{162} ^{163}IMNTFSVVPSPK^{174} ^{253}LAVNMVPFPR^{262} ^{263}LHFFMPGFAPLTSR^{276} ^{283}ALTVPELTQQVFDAK^{297}	Up
245	50	6.77	Aldehyde dehydrogenase E3′	^{3}AFEPATGR^{10} ^{289}IGDPLLEDTR^{298}	Up
342	40	7.68	Sialidase 2 (*N*-acetyl-α-neuraminidase)	^{10}ETLFQTGDYAYR^{21} ^{45}TDEHADLFVLR^{55} ^{244}VQAQSPNSGLDFQDNQVVSK^{263} ^{285}ADALDVWLLYTHPTDSR^{301}	Down
632	18	4.87	Ribosomal protein, large P2	^{3}VASYLLAALGGNSSPSAK^{21} ^{50}NIEDVIAQGVGK^{61}	Down

In confirmation of the Gα12 hypothesis, we observed a substantial increase in Gα12 expression in morphine-treated hMOR-CHO cells. As reported in Fig. 1, A and B, chronic morphine increased Gα12 (66%) expression in hMOR-CHO cells, but not in T394A-CHO cells, strongly suggesting that the observed up-regulation of Gα12 is related to the development of morphine tolerance and dependence in these cells. The fact that naloxone can block chronic morphine-induced up-regulation of Gα12 (Fig. 1C) further supports the link between morphine pretreatment and up-regulation of Gα12. It is now well established that the opioid μ receptor does not couple with the Gα12 protein under normal conditions (Connor and Christie, 1999), and our antibody capture data (Fig. 2) support this. Numerous studies document that the small GTPase RhoA is involved in the regulation of various cellular functions such as remodeling of the actin cytoskeleton and induction of transcriptional activity. Gα12/Gα13 are the major upstream regulators of RhoA activity. The thrombin receptor PAR-1 has been shown to couple to all three G protein families (Gα12/Gα13, Gaq, and Gai) and to regulate a substantial network of signaling pathways (Coughlin, 2000; Vogt et al., 2003). Moreover, Yamaguchi et al. (2003) reported that thrombin selectively activates RhoA activity via the α subunit of G12. Therefore, we determined the effects of thrombin on [^{35}S]GTPγS binding, RhoA activity (GTP-Rho pull-down), and Rho-dependent cytoskeletal responses in the control and morphine-treated hMOR-CHO cells since CHO cells are known to possess thrombin receptors (Majumdar et al., 2004).

Our results showed that chronic morphine had no effect on thrombin-stimulated [^{35}S]GTPγS binding (Fig. 3), indicating that chronic morphine did not alter the ability of thrombin to activate G proteins. Further examination of downstream effects showed that chronic morphine decreased the basal level of RhoA activity (Fig. 4A), and thrombin increased RhoA activity to about the same level as observed in control membranes, thereby enhancing stimulation of RhoA activity. This was likely due to the increased expression level of Gα12 in morphine-treated cells. The fact that thrombin did not increase α-actinin expression in untreated hMOR-CHO cells, despite an increase in RhoA activity, suggests that actual expression of the α-actinin protein is not tightly coupled to small changes in RhoA activity. It is possible, however, that the mRNA for α-actinin might have increased, but not enough to trigger a detectable increase in α-actinin expression. On the other hand, we speculate that, in the presence of an increased expression of Gα12 produced by chronic morphine treatment, the enhanced thrombin-stimulated RhoA activity was sufficient to significantly increase the expression level of α-actinin in morphine-treated cells (Fig. 4B). The results of proteomic analysis (see Table 2) support this idea since chronic morphine increased the expression of a number of proteins associated with morphological changes such as junction plakoglobin (a major cytoplasmic protein), tubulin β chain, and heat shock protein. Importantly, up-regulation of Gα12 and α-actinin by chronic morphine was also observed in mouse brain. Further functional assay of chronic morphine-induced changes in mouse brain will be needed to determine whether these changes correspond to the increased functional activity of the Gα12 system observed here in CHO

cells, such as thrombin-stimulated [^{35}S]GTPγS binding and thrombin-stimulated RhoA activity. In this regard, it should be noted that although we used thrombin as a model activator of the Gα12 system in CHO cells, the altered functional activity of Gα12 in mouse brain could also result either from constitutively active Gα12 or from other activated neurotransmitter receptors that couple with Gα12, such as endothelin receptors (Mao et al., 1998). Interestingly, several recent reports demonstrate functional interactions between the endothelin and opioid systems in rats (Wang et al., 2004).

In light of the well established role of Gα12 and RhoA in regulating cellular morphology, these data suggest that chronic morphine, in addition to promoting a wide range of cellular and molecular changes (Nestler and Aghajanian, 1997), might also promote morphological changes in neurons. Recent data demonstrating structural neuronal plasticity produced by exposure to drugs of abuse support this notion (Garcia-Sevilla et al., 2004; Marie-Claire et al., 2004; Robinson and Kolb, 2004). Repeated exposure to drugs of abuse (cocaine, amphetamine, nicotine, and morphine) produces persistent changes in the structure of dendrites and dendritic spines on cells in brain regions associated with incentive motivation, reward, and learning (Robinson and Kolb, 2004). Dendrites and dendritic spines are thought to be a locus of experience-dependent structural plasticity; therefore, a focus of much research on structural plasticity has been on the morphology of dendrites and dendritic spines. Viewed collectively, the present study provides further evidence that changes in the level and activity of G protein subunits regulate the functional responsiveness of G protein-coupled receptors and that neuroadaptation to chronic morphine treatment involves modifications of the expression and function of cytoskeletal proteins.

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