

# Long-Term Morphine Treatment Enhances Proteasome-Dependent Degradation of G $\beta$ in Human Neuroblastoma SH-SY5Y Cells: Correlation with Onset of Adenylate Cyclase Sensitization

Lionel Moulédous, Jérémie Neasta, Sandrine Uttenweiler-Joseph, Alexandre Stella, Mariette Matondo, Maïthé Corbani, Bernard Monsarrat, and Jean-Claude Meunier

*Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique, Toulouse, France*

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## ABSTRACT

The initial aim of this study was to identify protein changes associated with long-term morphine treatment in a recombinant human neuroblastoma SH-SY5Y clone (sc2) stably overexpressing the human  $\mu$ -opioid (MOP) receptor. In MOP receptor-overexpressing sc2 cells, short-term morphine exposure was found to be much more potent and efficacious in inhibiting forskolin-elicited production of cAMP, and long-term morphine exposure was shown to induce a substantially higher degree of opiate dependence, as reflected by adenylate cyclase sensitization, than it did in wild-type neuroblastoma cells. Differential proteomic analysis of detergent-resistant membrane rafts isolated from untreated and chronically morphine-treated sc2 cells revealed long-term morphine exposure to have reliably induced a 30 to 40% decrease in the abundance of five proteins, subsequently identified by mass spectrometry as G protein sub-

units  $\alpha_{i2}$ ,  $\alpha_{i3}$ ,  $\beta_1$ , and  $\beta_2$ , and prohibitin. Quantitative Western blot analyses of whole-cell extracts showed that long-term morphine treatment-induced down-regulation of G $\beta$  but not of the other proteins is highly correlated ( $r^2 = 0.96$ ) with sensitization of adenylate cyclase. Down-regulation of G $\beta$  and adenylate cyclase sensitization elicited by long-term morphine treatment were suppressed in the presence of carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (MG-115) or lactacystin. Thus, sustained activation of the MOP receptor by morphine in sc2 cells seems to promote proteasomal degradation of G $\beta$  to sensitize adenylate cyclase. Together, our data suggest that the long-term administration of opiates may elicit dependence by altering the neuronal balance of heterotrimeric G proteins and adenylate cyclases, with the ubiquitin-proteasome pathway playing a pivotal role.

It is widely agreed that dependence elicited by repeated drug use reflects neuronal changes that durably alter synaptic transmission within select neural circuits in the central nervous system (Koob et al., 1998; Kelly, 2004; Nestler, 2004). These plastic changes are likely to be similar to those underlying memory formation and storage, as has been doc-

umented in simple neuronal systems, with second messengers such as cAMP playing a key role (Kandel, 2001). Indeed, one fundamental neuronal change associated with long-term opiate exposure is “hypertrophy of the cAMP system” (Collier and Francis, 1975). Hypertrophy of the cAMP system may be brought about by a compensatory adaptation of neuronal adenylate cyclase (AC) to long-term opiate inhibition known as adenylate cyclase sensitization (ACS) (Thomas and Hoffman, 1987; Johnston and Watts, 2003).

Long-term treatment of many cell types with drugs such as morphine that inhibit AC induce enhanced activity of the enzyme after withdrawal of the inhibitory drug (Thomas and Hoffman, 1987). The phenomenon was first reported by Sharma et al. (1975a,b), who found that pretreatment of hybrid glioma X neuroblastoma NG 108–15 cells with mor-

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**ABBREVIATIONS:** AC, adenylate cyclase; ACS, adenylate cyclase sensitization; DRM, detergent-resistant membrane; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis; MG-115, carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal; wt, wild type; MOP,  $\mu$ -opioid; CHO, Chinese hamster ovary; TM, total membrane; KRH, Krebs-Ringer-HEPES; MES, 2-(N-morpholino)ethanesulfonic acid; MBS, modified Barth’s solution; Ro20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone.

phine for at least 12 h, upon removal of the drug, led to an increase in both basal and prostaglandin  $E_1$ -stimulated AC activity. The morphine-treated cells were considered to be dependent on morphine in the sense that they required the drug to maintain normal levels of AC activity and intracellular cAMP. The opiate-dependent status of these cells was characterized by an overshoot of cAMP upon withdrawal of the drug. ACS has since been observed in several other cell types, including human neuroblastoma SH-SY5Y cells (Yu et al., 1990) and transfected non-neuronal cells expressing the  $\mu$ -opioid (MOP) receptor (Avidor-Reiss et al., 1995). In MOP receptor-transfected CHO cells, the onset of ACS is independent of protein synthesis and does not occur in pertussis toxin-pretreated cells, indicating a requirement for the activation of a  $G_{i/o}$  type of heterotrimeric G protein (Avidor-Reiss et al., 1995). Moreover, in COS cells cotransfected with the MOP receptor and different AC isoenzymes, long-term morphine treatment is found to sensitize isoforms 1, 5, 6, and 8, with sensitization of isoform 5 being particularly marked, but not isoforms 2, 3, 4, and 7 (Avidor-Reiss et al., 1996, 1997).

There is evidence that long-term morphine treatment induces ACS in the brain as well. For example, levels of AC activity and protein kinase A, activation of which is a foreseeable consequence of hypertrophy of the cAMP pathway, have been shown to be increased in the brain of rats who received long-term treatment with morphine (Nestler and Tallman, 1988; Duman et al., 1988; Terwilliger et al., 1991; Maldonado et al., 1995). Likewise, gene disruption of two of the three types of cAMP response element-binding protein, activation of which is also expected to result from hypertrophy of the cAMP pathway, attenuates symptoms of morphine withdrawal in mice (Maldonado et al., 1996).

In the present study, we sought to identify protein changes elicited by long-term exposure of neuroblastoma cells to morphine and attempted to relate these changes to opiate dependence, as reflected by sensitization of adenylate cyclase. To this end, a recombinant human neuroblastoma SH-SY5Y subclone (sc2) stably overexpressing the human MOP receptor was used in which long-term morphine treatment induced a substantially higher degree of dependence than in wild-type cells. Protein changes elicited by long-term morphine exposure were sought by comparison of the subproteome of detergent-resistant membrane (DRM) raft preparations isolated from drug-naïve and continually morphine-treated sc2 cells. DRM rafts isolated from various cell types are indeed enriched in signaling proteins, including heterotrimeric G proteins (von Haller et al., 2001; Foster et al., 2003; Blonder et al., 2004), and there is mounting evidence that G protein-coupled receptor-mediated transmembrane signaling is spatially confined to these membrane microdomains (Cooper, 2003; Chini and Parenti, 2004; Ostrom and Insel, 2004). In addition, the raft proteome must be composed of considerably fewer protein species than the whole-cell proteome, thus allowing for a more quantitative analysis. Therefore, we expected that protein changes elicited by long-term morphine exposure in sc2 cells would be more readily detected in the raft subproteome than in the whole-cell proteome. Indeed, quantitative proteomic analysis of DRM rafts isolated from untreated and continually morphine-treated SH-SY5Y sc2 cells revealed long-term morphine exposure to have induced a 30 to 40% decrease in the abundance of several proteins, subsequently identified by mass spectrometry as G protein

subunits  $\alpha_2$ ,  $\alpha_3$ ,  $\beta_1$ , and  $\beta_2$ , and prohibitin. Down-regulation of G $\beta$  but not of the other proteins is strictly correlated with the amplitude of ACS elicited by continually applied morphine, suggestive of a cause-and-effect relationship. Furthermore, down-regulation of G $\beta$  elicited by long-term morphine treatment is shown to be totally suppressed in the presence of MG-115 or lactacystin. These data suggest that in sc2 cells, sustained activation of the MOP receptor may sensitize adenylate cyclase by promoting proteasomal degradation of G $\beta$ . Most importantly, they identify the ubiquitin-proteasome pathway as being potentially involved in opiate dependence.

## Materials and Methods

**Cell Culture and Transfection.** SH-SY5Y neuroblastoma cells were transfected with N-terminal T7-tagged human  $\mu$ -opioid receptor in pRC-CMV using Lipofectamine. Stably transfected G418-resistant cells were grown in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, 50  $\mu$ g/ml gentamicin, and 400  $\mu$ g/ml G418 (all from Invitrogen) in 5%  $CO_2$  at 37°C.

**Membrane Preparation.** Cells were harvested in ice-cold phosphate-buffered saline, frozen at  $-70^\circ C$  for 1 h, thawed, and homogenized in 50 mM Tris-HCl, pH 7.4, in a Potter Elvehjem tissue grinder (VWR Int., Fontenay-sous-Bois, France). The nuclear pellet was discarded by centrifugation at 1000g for 10 min, and the total membrane (TM) fraction was collected upon centrifugation at 100,000g for 35 min. Protein concentration was determined by the method described by Lowry et al. (1951), with bovine serum albumin used as standard.

**Measurement of Opiate Binding Sites.** Opiate binding sites were assayed for the TM fraction from neuroblastoma cell by using [ $^3H$ ]diprenorphine (50 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA). Membranes (10–60  $\mu$ g of protein) were incubated for 1 h at 25°C with increasing concentrations of tritiated ligand in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4. Nonspecific binding was determined in the presence of 1  $\mu$ M concentration of unlabeled diprenorphine. Bound radioligand was collected by filtration on glass-fiber filters (GF/B; Whatman, Clifton, NJ), and radioactivity counting was performed in a Packard model 2100TR liquid scintillation analyzer (PerkinElmer).

**Measurement of Intracellular cAMP.** Twenty-four-well plates were seeded with  $\sim 2 \times 10^5$  SH-SY5Y cells in culture medium and incubated for approximately 16 h at 37°C. The culture medium was then replaced with fresh medium containing the various agents morphine sulfate (Francopia, Paris, France), MG-115, or lactacystin (Sigma Chemical Co., St. Louis, MO), or vehicle. After incubation for the desired time, the culture medium was removed, and 300  $\mu$ l of fresh medium was added containing 0.1  $\mu$ M adenine and 1  $\mu$ Ci [ $^3H$ ]adenine (24 Ci/mmol; Amersham Biosciences, Piscataway, NJ) with or without morphine. After 1 h at 37°C, the cells were rinsed four times with 500  $\mu$ l of HEPES-buffered Krebs-Ringer saline (KRH; 124 mM NaCl, 5 mM KCl, 1.25 mM  $MgSO_4$ , 1.5 mM  $CaCl_2$ , 1.25 mM  $KH_2PO_4$ , 25 mM HEPES, 8 mM glucose, and 0.5 mg/ml bovine serum albumin, pH 7.4), and 200  $\mu$ l of fresh KRH was added to each well. Intracellular accumulation of cAMP was initiated by the addition of 100  $\mu$ l of KRH containing 5  $\mu$ M forskolin (Sigma), 0.1 mM 3-isobutyl-1-methylxanthine (Sigma), 0.1 mM Ro20-1724 (BIOMOL Research Laboratories, Plymouth Meeting, PA). After exactly 10 min at 37°C, the reaction was stopped by the addition of 30  $\mu$ l of HCl 2.2 M and rapid mixing. The [ $^3H$ ]cAMP content was determined by selective batch elution on acidic alumina columns, essentially as described by Alvarez and Daniels (1992).

**Isolation of DRM Fraction.** Cells ( $\sim 8 \times 10^7$  per gradient) were harvested in ice-cold phosphate-buffered saline, frozen at  $-70^\circ C$  for 1 h, and resuspended in 1.7 ml of MBS [25 mM MES and 0.15 M NaCl] containing proteinase inhibitors (Complete Mini tablets;

Roche Diagnostics, Indianapolis, IN) and 1% Triton X-100 (Sigma). After 20 min of gentle agitation at 4°C, the lysate was mixed with an equal volume of ice-cold 80% sucrose in MBS buffer. Of the 40% sucrose/cell lysate solution, 3 ml was transferred to 12-ml Polyallomer centrifuge tubes (Beckman Coulter, Fullerton, CA) on ice, overlaid successively with 6 ml of 30% sucrose and 2.5 ml of 5% sucrose in MBS, and centrifuged at 200,000g for 18 h at 4°C in a Kontron Ultracentrifuge TGA-65. Twelve 1-ml fractions were collected from the top down. Alkaline phosphatase activity was measured in 50  $\mu$ l of each fraction using FAST *p*-nitrophenyl phosphate tablet sets (Sigma). Fractions with the highest alkaline phosphatase activity (fractions 3 and 4 corresponding to low-density DRMs) were pooled, mixed with 22 ml of 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and recentrifuged at 100,000g for 2.3 h at 4°C. The DRM pellet was resuspended in 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and protein concentration was determined using the Lowry assay.

**Gel Electrophoresis and Image Analysis.** DRM proteins were solubilized in 1 $\times$  Laemmli sample buffer containing 5%  $\beta$ -mercaptoethanol, boiled for 5 min at 100°C, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gels (16  $\times$  16 cm, 1.5 mm thick) using a Hoefer SE 600 Ruby device. Gels were stained with colloidal Coomassie blue [17% (w/v) ammonium sulfate, 34% (v/v) methanol, 3% (v/v) orthophosphoric acid, and 0.1% (w/v) brilliant blue G-250] for 24 h. Gels were scanned using a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA) and analyzed using Quantity One software (Bio-Rad). Band size was calculated as the area under the intensity profile curve after background subtraction. To account for possible variation in protein load, Student's *t* test statistical analysis was performed on normalized band densities with reference to the summed band density for the whole lane. Two other normalization methods, such as expression of band density either as a fraction of the total density for the lane or relative to the density of a single reference band, gave similar results.

**Western Blot Analysis.** Samples were solubilized in 1 $\times$  SDS-PAGE sample buffer containing 5%  $\beta$ -mercaptoethanol by boiling for 5 min at 100°C. Proteins were subjected to SDS-PAGE followed by liquid transfer on nitrocellulose membranes (Hybond-C Extra; Amersham). The following antibodies were used for immunoblotting: monoclonal anti-flotillin 1 (Transduction Laboratories, Lexington, KY) and anti-T7 Tag (Novagen, Madison, WI), polyclonal anti-actin (Sigma), polyclonal anti-G $\alpha$ i (C-10, reactive with G $\alpha$ i1, G $\alpha$ i2, and G $\alpha$ i3 subunits; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-G $\beta$  (T-20, reactive with G $\beta$ 1, G $\beta$ 2, G $\beta$ 3, and G $\beta$ 4 subunits; Santa Cruz Biotechnology). After enhanced chemiluminescence revelation (Amersham), X-ray films were scanned using a GS-800 Calibrated Densitometer (Bio-Rad). When required, blots were quantified using Quantity One software (Bio-Rad) relative to the actin used as internal standard.

**In-Gel Protein Digestion.** The gel pieces were washed and dried under vacuum. Proteins were reduced and alkylated by rehydration with 10 mM dithiothreitol in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> for 35 min at 56°C followed by incubation in 55 mM iodoacetamide in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> for 30 min at room temperature in the dark. Gel pieces were then washed with 0.1 M NH<sub>4</sub>HCO<sub>3</sub> and acetonitrile and dried in a vacuum centrifuge. Gel pieces were rehydrated in a sufficient covering volume of modified trypsin solution (12.5 ng/ $\mu$ l in 50 mM NH<sub>4</sub>HCO<sub>3</sub>; Promega, Madison, WI) and incubated overnight at 37°C. Before peptide extraction, 0.5  $\mu$ l of the tryptic digest was spotted onto the MALDI target plate. Peptides were extracted three times at 37°C for 15 min with shaking, once using 50 mM NH<sub>4</sub>HCO<sub>3</sub> and twice using 5% formic acid in 50% acetonitrile. The peptide mixture was concentrated in a vacuum centrifuge to a final volume of 10  $\mu$ l.

**Mass Spectrometry Analysis and Database Search.** MALDI-TOF mass spectrometry (MS) analyses were performed on a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA). A 0.5- $\mu$ l sample of tryptic digest supernatant was applied on the MALDI target plate with 0.3  $\mu$ l of matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid; 8 mg/ml in H<sub>2</sub>O/acetonitrile/trifluoro-

acetic acid, 50:50:0.1). Mass spectra were acquired in an automated positive reflector mode, from *m/z* 700 to *m/z* 3500, and calibrated with autodigestion trypsin peaks. Peak lists from peptide mass mapping spectra were automatically extracted from the data stored in an Oracle database and electronically submitted for searching against human sequences in the Swiss-Prot and the National Center for Biotechnology Information databases, using Mascot software (Matrix Science, Boston, MA). Protein identification was systematically confirmed by MS/MS analyses. Tryptic peptide extracts were subjected to nanoLC-MS/MS analysis on an ESI-Qq-TOF mass spectrometer (QSTAR Pulsar; Applied Biosystems) operating in positive mode with a 2.1-kV spray voltage. Chromatographic separation was performed onto a 75- $\mu$ m internal diameter  $\times$  15 cm PepMap C18 precolumn using a linear gradient of increasing acetonitrile in water (5–50%) over 40 min with 0.1% formic acid as an ion-pairing agent. MS and MS/MS data were recorded continuously with a 5-s cycle time. Within each cycle, MS data were accumulated for 1 s followed by two MS/MS acquisitions of 2 s each on the two most abundant ions. Dynamic exclusion was used within 30 s to prevent repetitive selection of the same ions. Collision energies were automatically adjusted according to the charge state and mass value of the precursor ions. Mascot software was used for protein identification by searching in the Swiss-Prot and National Center for Biotechnology Information databases with MS/MS spectra. Species were restricted to human.

**Data Analysis.** Experimental data-fitting and statistical analysis were performed using Prism (GraphPad Software Inc., San Diego, CA). Statistical significance between groups of data was assessed using either Student's *t* test or one-way ANOVA followed by the Tukey post hoc test.

## Results

**Short- and Long-Term Effects of Morphine on Forskolin-Stimulated cAMP Production in Wild-Type and MOP Receptor-Overexpressing SH-SY5Y Clones.** Because our own preliminary studies had shown that wild-type (wt), undifferentiated SH-SY5Y neuroblastoma cells responded poorly to both short- and long-term exposure to morphine, we generated transfected clones overexpressing the human MOP receptor.

The parent (wt) neuroblastoma SH-SY5Y clone, which expresses 0.37  $\pm$  0.01 pmol opiate binding sites/mg membrane protein, and two selected genetically engineered subclones thereof, sc4 and sc2, which express, respectively, 0.60  $\pm$  0.04 and 2.2  $\pm$  0.3 pmol opiate binding sites/mg membrane protein, were tested for short-term morphine exposure inhibition of forskolin-stimulated production of cAMP. Morphine was found to be far more potent and efficacious in inhibiting forskolin-stimulated production of cAMP in sc4 and sc2 cells than in wt cells (Fig. 1a). In wt cells, 10  $\mu$ M morphine, the highest concentration tested, elicited less than 30% inhibition, whereas the opiate inhibited forskolin-stimulated production of cAMP completely ( $E_{\text{max}}$  = 98  $\pm$  2%), with half-maximal inhibition being attained with 4  $\pm$  0.6 nM morphine in sc2 cells. sc4 cells displayed an intermediate response toward morphine with maximum inhibition being 88  $\pm$  2%, with half-maximum inhibition at 31  $\pm$  5 nM morphine.

Likewise, long-term morphine exposure elicited greater ACS—as assessed by forskolin-stimulated cAMP production after removal of the drug—in sc4 and sc2 cells than in wt cells (Fig. 1b). An 18-h exposure to 10  $\mu$ M morphine was found to increase forskolin-stimulated cAMP production 1.31  $\pm$  0.42-fold in wt cells, 1.51  $\pm$  0.11-fold in sc4 cells, and 2.56  $\pm$  0.50-fold in sc2 cells. This effect was independent of

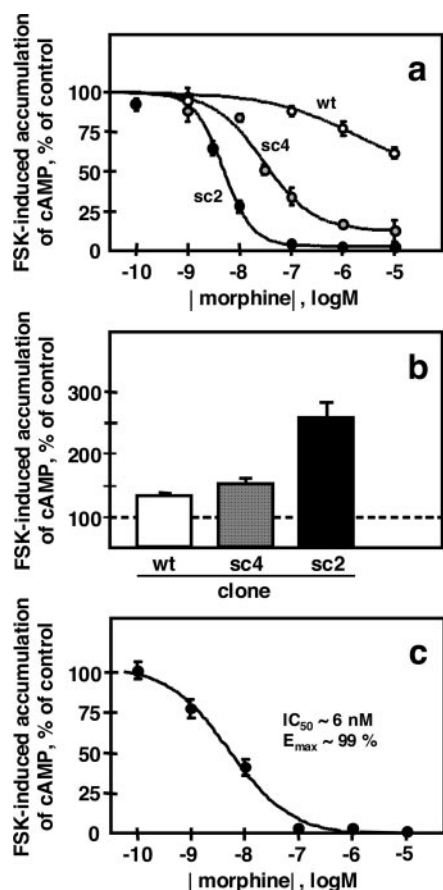
protein synthesis (i.e., resisted cycloheximide treatment) and did not occur in pertussis toxin-pretreated cells (data not shown). Thus, in neuroblastoma cells, there seems to be a positive correlation between the level of expressed MOP receptor and the ability of not only short-term morphine exposure to inhibit AC activity but also long-term morphine exposure to induce ACS, a hallmark of opiate dependence.

In sc2 cells continually treated with morphine, the sensitized AC activity remained as potently and efficaciously inhibited by morphine as in drug-naïve cells. As Fig. 1c shows, the opiate acutely inhibited sensitized AC activity with  $EC_{50}$  and  $E_{max}$  values, respectively, of  $5.9 \pm 1.2$  nM and  $99.2 \pm 0.6\%$ , similar to values ( $4 \pm 0.6$  nM and  $98 \pm 2\%$ ) obtained in

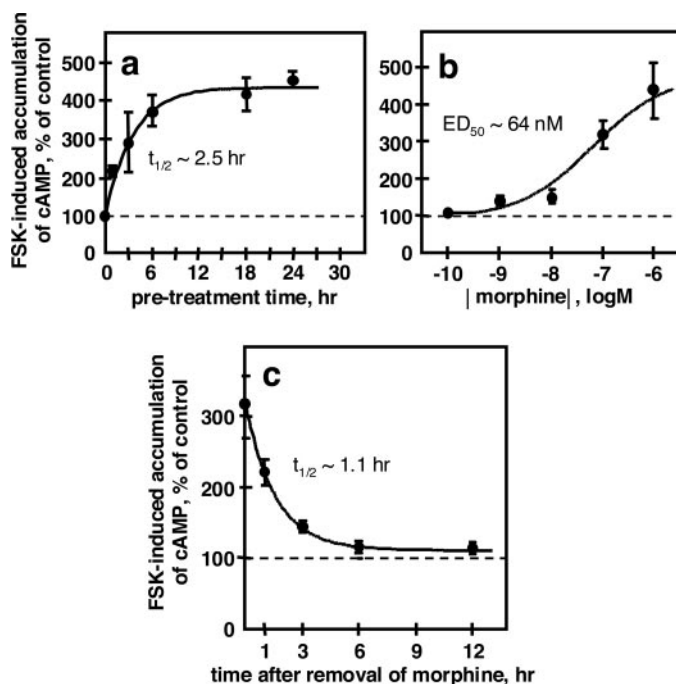
untreated cells. This showed that morphine did not induce desensitization of the MOP receptor in SH-SY5Y cells. It also indicated that inhibition of the sensitized AC activity by morphine uses the same molecular pathway (i.e., the MOP receptor and a heterotrimeric  $G_{i/o}$  protein), as does inhibition of the nonsensitized AC activity.

**Time Course, Dose-Dependence, and Reversibility of Morphine-Induced ACS in SH-SY5Y sc2 Cells.** Because it responded maximally, particularly in terms of ACS elicited by long-term morphine exposure, the MOP receptor-overexpressing SH-SY5Y subclone 2 was selected for further investigation. ACS elicited by treatment of sc2 cells with  $1 \mu\text{M}$  morphine was time-dependent and developed slowly (Fig. 2a). Half-maximum sensitization was reached after  $2.5 \pm 0.4$  h. Maximum sensitization required that the cells be exposed to  $1 \mu\text{M}$  morphine for at least 12 h and amounted to approximately 4.5-fold.

ACS elicited by treatment of sc2 cells with morphine for 18 h was dose-related (Fig. 2b), with half-maximum sensitization, in this case approximately 2.15-fold, being observed in the presence of  $64 \pm 18$  nM morphine. This is a substantially higher concentration than that ( $4 \pm 0.6$  nM) causing



**Fig. 1.** Short- and long-term effects of morphine on forskolin-stimulated production of cAMP in human MOP receptor-overexpressing versus wild-type neuroblastoma SH-SY5Y clones. **a**, short-term morphine exposure is far more potent and efficacious in inhibiting forskolin-induced cAMP production in SH-SY5Y subclones 2 (sc2) and 4 (sc4) than in parent (wt) SH-SY5Y cells. The cells were incubated for 10 min with  $5 \mu\text{M}$  forskolin and morphine at the indicated concentration and were assayed for cAMP content as described under *Materials and Methods*. Control refers to forskolin-stimulated cAMP production in the absence of morphine. **b**, long-term morphine exposure induces a substantially higher degree of adenylate cyclase sensitization in SH-SY5Y subclones 2 (sc2) and 4 (sc4) than in parent (wt) SH-SY5Y cells. Here, the cells were incubated with  $10 \mu\text{M}$  morphine for 18 h, rapidly washed free of drug, and assayed for forskolin-stimulated cAMP production. Control refers to cells that had not been exposed to morphine. **c**, morphine-conditioned SH-SY5Y sc2 cells show unchanged response to short-term morphine exposure (compare with sc2 curve in **a**). Cells were incubated with  $1 \mu\text{M}$  morphine for 6 h, washed free of drug, and assayed for inhibition of forskolin-stimulated cAMP production by morphine at the indicated concentration. Control refers to cells not exposed to morphine. Data are expressed as mean  $\pm$  S.E.M. of at least three independent experiments with each measurement performed in triplicate.



**Fig. 2.** AC sensitization elicited by long-term morphine exposure in SH-SY5Y sc2 cells is time-dependent, dose-related, and reversible. **a**, the time course of AC sensitization was determined by incubating the cells with  $1 \mu\text{M}$  morphine for the indicated period of time, removal of the drug, and assay of forskolin-stimulated cAMP production as described under *Materials and Methods*. Half-maximum sensitization was attained approximately 2.5 h after exposure to morphine. **b**, dose-dependence of AC sensitization was determined by incubating cells for 18 h with morphine at the indicated concentration, washing free of the drug, and assaying for forskolin-stimulated cAMP production as described under *Materials and Methods*. Under these conditions, the concentration of morphine that caused half-maximum sensitization was estimated to be approximately 60 nM. **c**, the reversibility of AC sensitization was determined by incubating cells for 24 h with  $1 \mu\text{M}$  morphine, drug removal, and assay of forskolin-stimulated cAMP production at the indicated time after drug removal. The AC-sensitized state waned completely within 6 h, with half-maximum desensitization being observed after approximately 1 h. Controls in **a**, **b**, and **c** refer to cells not exposed to morphine. Data are expressed as the mean  $\pm$  S.E.M. of at least three independent experiments with each measurement performed in triplicate.

half-maximum short-term inhibition of forskolin-stimulated cAMP production in these cells.

Finally, ACS elicited by long-term exposure to morphine was reversible (Fig. 2c) and diminished slowly upon drug removal. Challenging morphine-treated sc2 cells with forskolin at increasing time intervals after drug removal resulted in progressively decreased forskolin-stimulated production of intracellular cAMP, which returned to near control levels within approximately 6 h. The half-life of ACS elicited by a 24-h exposure to 1  $\mu$ M morphine was estimated to be 1.1 h.

**Identification of Long-Term Morphine-Responsive Proteins in SH-SY5Y sc2 Cells.** A proteomics approach limited to the proteome of detergent-resistant membrane rafts was implemented (see Introduction) to identify proteins that might have undergone changes in abundance or location in response to long-term morphine exposure in sc2 cells.

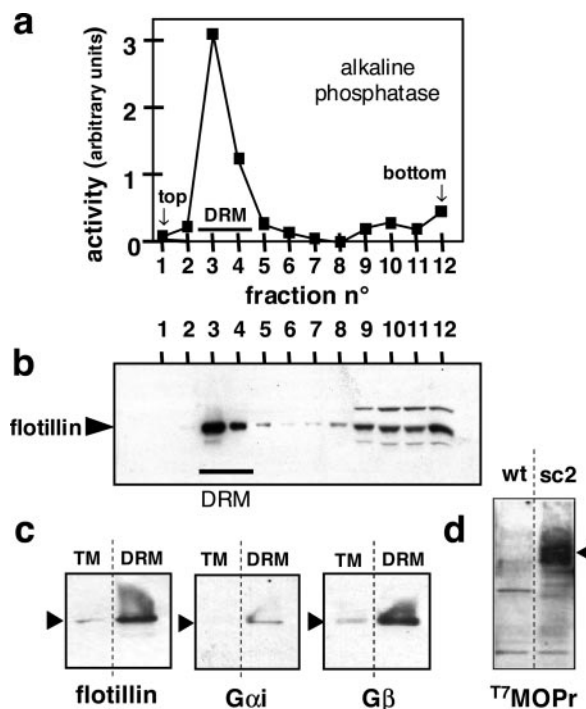
Membrane rafts were prepared from intact sc2 cells as a buoyant membrane fraction that is insoluble in cold Triton X-100 (1%, v/v), the so-called DRM fraction. This fraction, recovered at the 30/5% sucrose interface is enriched in specific raft markers, in this case alkaline phosphatase (Fig. 3a), a glycosylphosphatidylinositol-anchored protein, and flotillin (Fig. 3, b and c, left). It is also enriched in the G $\alpha$ i and G $\beta$  subunits of heterotrimeric G proteins (Fig. 3c, middle and right) and contains the T7 epitope-tagged MOP receptor (Fig. 3d). This is consistent with the notion that morphine signaling may occur, at least in part, within DRM rafts in sc2 cells.

The DRM fraction isolated from untreated, briefly (15 min) morphine-treated, and continually (6 and 24 h) morphine-treated sc2 cells was solubilized with SDS, submitted to one-dimensional PAGE, and the proteins were stained with colloidal Coomassie blue. Figure 4a shows a representative one-dimensional protein map of the DRM fraction from neuroblastoma sc2 cells composed of at least 80 distinct protein bands.

Quantitative densitometric analyses of the stained gels indicated that short-term morphine exposure did not induce qualitative or quantitative detectable change in the rafts proteome from neuroblastoma cells (data not shown). In contrast, five protein bands (annotated I to V in Fig. 4a) were found to have been significantly down-regulated in rafts from continually morphine-treated cells. The identity of the protein(s) contained in these bands was determined by peptide mass fingerprinting using MALDI-MS and partial sequencing using nano-liquid chromatography-electrospray ionization-MS/MS, as described under *Materials and Methods*. Band I/II was found to contain heterotrimeric G protein subunits  $\alpha$ i2 and  $\alpha$ i3, bands III and IV, heterotrimeric G protein subunits  $\beta$ 1 and  $\beta$ 2, respectively, and band V, prohibitin (Fig. 4b). It is noteworthy that the down-regulated G protein subunits are established inhibitors of AC, especially isoform i, which is expressed in neuroblastoma SH-SY5Y cells (see *Discussion*). A 6-h pretreatment with morphine resulted in a significant decrease in the relative amount of G $\beta$ 1 ( $-16.1 \pm 2.7\%$ ;  $p = 0.0236$ ) but not of G $\alpha$ i2, G $\alpha$ i3, G $\beta$ 2, or prohibitin. A 24-h pretreatment with morphine caused a significant reduction in the relative amounts of the five proteins G $\alpha$ i2 ( $-16.8 \pm 2.3\%$ ;  $p = 0.0423$ ), G $\alpha$ i3 ( $-25.9 \pm 1.6\%$ ;  $p = 0.0175$ ), G $\beta$ 1 ( $-24.1 \pm 6.2\%$ ;  $p = 0.0246$ ), G $\beta$ 2 ( $-34.1 \pm 5.6\%$ ;  $p = 0.0148$ ) (Fig. 4c), and prohibitin ( $-38.4 \pm 6.4\%$ ;  $p = 0.0464$ ).

Down-regulation of the G protein subunits elicited by long-

term morphine treatment was confirmed by Western blot analysis of whole neuroblastoma cell extracts using antibodies to G $\alpha$ i and G $\beta$ . Figure 5 shows that in comparison with no pretreatment, pretreatment of the cells with morphine for 6 h induced a significant decrease in the amount of G $\beta$  ( $-28.5 \pm 6.3\%$ ,  $p = 0.004$ ) but no significant change in the amount of G $\alpha$ i ( $8.9 \pm 9.5\%$ ;  $p = 0.3816$ ). Pretreatment of the cells with morphine for 24 h resulted in further down-regulation of G $\beta$  ( $-35.6 \pm 6.1\%$ ,  $p = 0.0012$ ) and substantial down-regulation of G $\alpha$ i ( $-35.7 \pm 4.9\%$ ,  $p = 0.0003$ ). Down-regulation of G $\beta$  but not G $\alpha$ i (or prohibitin) was apparent after 6 h of exposure to morphine, a condition producing submaximal ACS in sc2 cells (Fig. 2a), making it clear that down-regulation of G $\beta$  but not G $\alpha$ i (or prohibitin) could be correlated to ACS in SH-SY5Y sc2 cells. The remainder of the study therefore focused specifically on G $\beta$ .



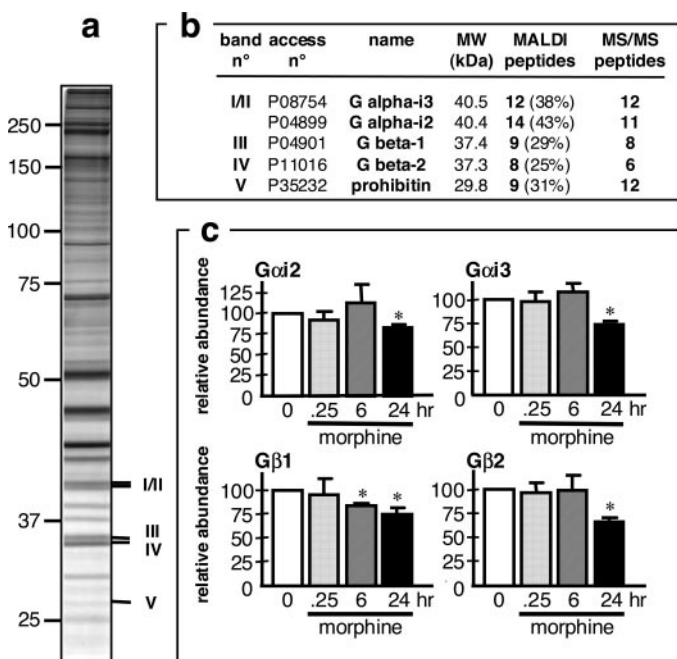
**Fig. 3.** Isolation of DRM rafts from SH-SY5Y sc2 cells. Cells were lysed in cold Triton X-100, and the lysate was floated by ultracentrifugation in a discontinuous sucrose density gradient as described under *Materials and Methods*. Each 1-ml gradient fraction was probed for the presence of alkaline phosphatase and flotillin, established membrane rafts markers. a, assay of 50- $\mu$ l aliquots of each fraction for alkaline phosphatase activity indicates that the glycosylphosphatidylinositol-anchored enzyme is recovered for the most part in the low-density fraction (labeled DRM) at the 30/5% sucrose interface near the top of the gradient. Much less activity is detected in the detergent-solubilized fractions at the bottom of the gradient. b, low-density fractions 3 and 4 (labeled DRM) at the 30/5% sucrose interface are enriched in flotillin. A 20- $\mu$ l aliquot of each gradient fraction was submitted to SDS-PAGE, transferred to nitrocellulose, and probed for flotillin-1 using a specific antibody. c, in comparison with the TM fraction (the high-speed pellet from whole-cell homogenates), the DRM fraction seems to be substantially enriched in flotillin (left). Equal amounts (10  $\mu$ g) of protein from pooled gradient fractions 3 and 4 (DRM fraction) and the TM fraction were submitted to SDS-PAGE and immunoblotted using an antibody to flotillin-1 (left). The TM and DRM fractions were also compared for heterotrimeric G protein subunits  $\alpha$ i (middle) or G $\beta$  (right) content by immunoblotting with the appropriate antibody, as described above. As is observed for flotillin, the DRM fraction is substantially enriched in G $\alpha$ i and G $\beta$  compared with the TM fraction. d, the T7-epitope tagged MOP receptor is present in the DRM fraction isolated from SH-SY5Y sc2 cells but not in that from wt cells, used as control. Immunoblotting was with an anti-T7 antibody.

**Correlation between Levels of G $\beta$  and ACS in SH-SY5Y sc2 Cells.** A high degree of correlation existed between levels of G $\beta$  and the amplitude of ACS elicited by long-term morphine treatment in SH-SY5Y sc2 cells. Quantitative Western blot analysis showed that down-regulation of G $\beta$  closely paralleled ACS with respect to both the duration of exposure to morphine and the morphine dose. Half-maximum down-regulation of G $\beta$  was attained after approximately 2 h of treatment with 1  $\mu$ M morphine (Fig. 6a), a duration close to that (2.5 h) necessary to produce half-maximum ACS (Fig. 2a). Likewise, the concentration of morphine eliciting half-maximum down-regulation of G $\beta$  (Fig. 6b) was calculated to be approximately 50 nM, similar to that (64 nM) producing half-maximum ACS (Fig. 2b) under the same conditions. Linear regression analysis of normalized experimental data shown in Figs. 2, a and b, and 6, a and b,

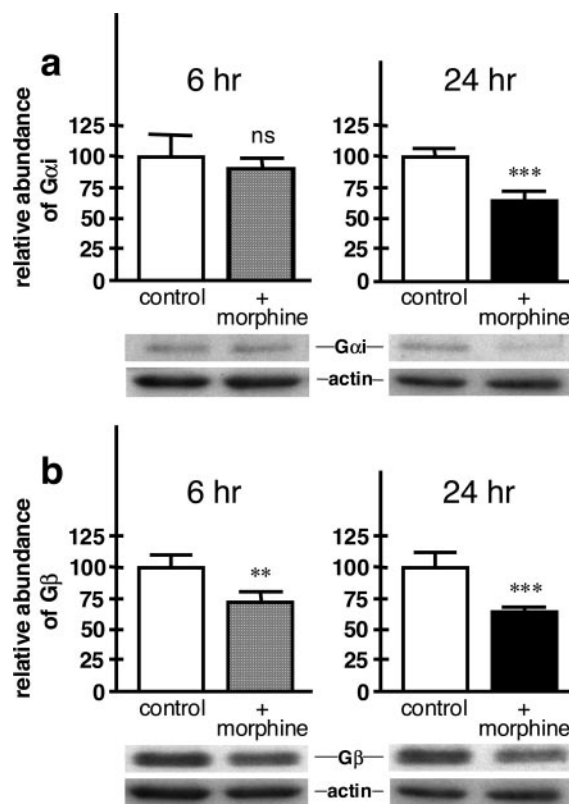
reveals the high correlation ( $r^2 = 0.9619$ ) between ACS and down-regulation of G $\beta$  (Fig. 6c), a strong indication that morphine-elicited down-regulation of G $\beta$  is involved in AC sensitization. On the other hand, the decrease in ACS observed in morphine-treated cells after removal of the drug was paralleled by an up-regulation of G $\beta$ , with cellular levels returning to control (untreated cells) values 6 h after withdrawal (i.e., when ACS was no longer present) (Fig. 6d).

**Effects of Inhibiting Proteasome Activity on Morphine-Induced Down-Regulation of G $\beta$  and ACS in SH-SY5Y sc2 Cells.** Because the ubiquitin-proteasome pathway provides one potential mechanism of G protein degradation (see *Discussion*), we tested whether blocking proteasome activity could affect long-term morphine-induced down-regulation of G $\beta$  and ACS in SH-SY5Y sc2 cells. sc2 cells were therefore treated with MG-115 or lactacystin, two inhibitors of proteasome activity, for 6 h in the absence or presence of morphine.

Treating sc2 cells with either proteasome inhibitor alone



**Fig. 4.** Identification of morphine-responsive proteins in detergent-resistant membrane rafts isolated from SH-SY5Y sc2 cells. a, representative one-dimensional (SDS-PAGE) protein map of DRM rafts isolated from SH-SY5Y sc2 cells. Staining was with colloidal Coomassie blue and revealed up to 80 distinct protein bands. Marker relative molecular mass ( $M_r$ ) is indicated to the left (in kilodaltons). The position of the five continually morphine-responsive (down-regulated) proteins is indicated in Roman numerals to the right. b, MALDI-MS and ESI-MS/MS protein identification reveals band(s) I/II to contain heterotrimeric G protein subunits  $\alpha$ 2 and  $\alpha$ 3, bands III and IV, heterotrimeric G protein subunits  $\beta$ 1 and  $\beta$ 2, respectively, and band V, prohibitin. MALDI peptides: the number of assigned peptides with  $M_r(\text{expt}) - M_r(\text{calc}) < 20$  ppm and 0 or 1 trypsin miscleavage. The percentage of the full-length sequence covered by the matching peptides is indicated in parentheses. MS/MS peptides: the number of peptides for which the spectrum allowed protein identification with Mascot individual ion scores  $> 28$ , indicating that the match is statistically significant ( $p < 0.05$ ). The accession number is that of the Swiss-Prot database. c, down-regulation of Gai2, Gai3, G $\beta$ 1, and G $\beta$ 2 elicited by long-term morphine exposure is time-dependent. SH-SY5Y sc2 cells were exposed to 1  $\mu$ M morphine for 0.25 (15 min), 6, or 24 h, and the DRM rafts were isolated and processed for densitometric quantification of the four morphine-responsive proteins as described under *Materials and Methods*. Note that only G $\beta$ 1 is significantly down-regulated after 6-h exposure to morphine, a condition that produces submaximal AC sensitization in sc2 cells (Fig. 2a). Unfilled bars represent the amount of protein in DRM rafts from cells not exposed to morphine. Data are expressed as mean  $\pm$  S.E.M. of at least four separate experiments. \*,  $p < 0.05$ .



**Fig. 5.** Long-term morphine exposure down-regulates heterotrimeric G protein  $\alpha$  and  $\beta$  subunits in SH-SY5Y sc2 cells: validation by quantitative immunoblot analysis in whole-cell extracts. a, SH-SY5Y sc2 cells were exposed to 1  $\mu$ M morphine for 6 (left) or 24 h (right) and were processed for SDS-PAGE and immunoblotting using an antibody to Gai1-3, as described under *Materials and Methods*. Significant down-regulation ( $-35.7 \pm 4.9\%$ ) of Gai was observed after 24 h but not after 6 h of treatment, results similar to those observed by densitometric analysis (Fig. 4c). b, same as in a, except that immunoblotting was with an antibody to G $\beta$ 1-4. Significant down-regulation ( $-28.5 \pm 6.3\%$ ) is manifest after a 6-h treatment with morphine, confirming the densitometric analysis (Fig. 4c). Treatment (24 h) with morphine (right) induced  $35.6 \pm 6.1\%$  down-regulation of G $\beta$ . Gai and G $\beta$  protein abundance was determined relative to an actin (morphine-unresponsive) internal standard. Unfilled bars represent the amount of protein in extracts from cells not exposed to morphine. Data are expressed as mean  $\pm$  S.E.M. of at least four separate experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns, not significant ( $p > 0.05$ ).

was found to enhance forskolin-stimulated synthesis of intracellular cAMP (Fig. 7a, left). However, treatment with proteasome inhibitor alone did not result in any significant change in G $\beta$  abundance, as assessed by quantitative Western blot analysis of whole-cell extracts (Fig. 7a, right). Thus, in contrast to ACS induced by long-term morphine treatment, ACS elicited by the proteasome inhibitors seemed to be independent of G $\beta$ . We verified (data not shown) that neither MG-115 nor lactacystin enhanced incorporation of [ $^3$ H]adenine into the intracellular ATP pool (see *Materials and Methods*) or induced up-regulation of the AC stimulatory G $\alpha$ s subunit.

Long-term exposure of sc2 cells to morphine in the presence of MG-115 or lactacystin did not produce any further enhancement of forskolin-stimulated production of cAMP over that elicited by either proteasome inhibitor alone (Fig.

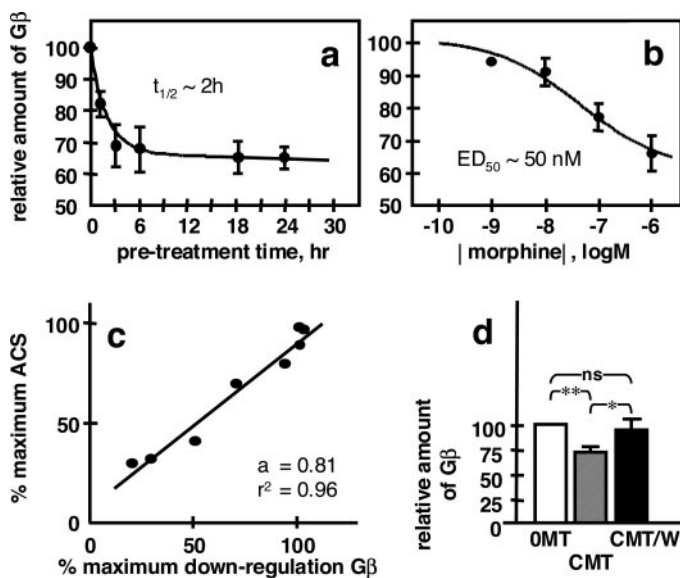
7b, left). This could be taken to mean that proteasome inhibition suppressed the ability of morphine to promote AC sensitization, although it cannot be excluded that the proteasome inhibitors prohibit further morphine sensitization by locking the enzyme into a maximally sensitized state. We were unable to identify experimental conditions, such as dose and/or duration of treatment with proteasome inhibitor, whereby ACS induced by proteasome inhibitor and morphine could be dissociated. However, whereas long-term morphine treatment induced substantial down-regulation of G $\beta$  in the absence of proteasome inhibitors, it did not so in the presence of MG-115 or lactacystin (Fig. 7b, right). The failure of long-term morphine to induce G $\beta$  down-regulation in MG-115- or lactacystin-treated cells provides a clear indication that down-regulation of G $\beta$  is brought about by proteasomal degradation of the G protein subunit.

## Discussion

The primary aim of this study was to identify protein changes elicited by long-term morphine treatment in an improved cellular model of opiate dependence [i.e., a recombinant human neuroblastoma SH-SY5Y clone (sc2)] stably overexpressing the MOP receptor, the principal if not exclusive vector of short- and long-term morphine actions in vivo (Matthes et al., 1996). In sc2 cells, morphine is shown to be far more potent and efficacious in acutely inhibiting forskolin-induced cAMP production, and long-term morphine exposure is shown to induce a degree of ACS, a hallmark of opiate dependence, substantially higher than that observed in wild-type cells. On the basis of mRNA content, SH-SY5Y cells are expected to express AC1 and AC8 (Jang and Juhn, 2001), two of the four AC isoforms shown previously to be sensitized upon long-term morphine treatment (Avidor-Reiss et al., 1997). However, it remains to be firmly established which particular AC isoform(s) morphine sensitizes in the neuroblastoma SH-SY5Y clone used in the present study.

Our proteomics analysis in which the proteomes of detergent-resistant membrane rafts isolated from drug-naïve and morphine-treated cells were compared has detected the significant down-regulation of five proteins, subsequently identified by mass spectrometry to be the heterotrimeric G protein subunits  $\alpha$ 2,  $\alpha$ 3,  $\beta$ 1, and  $\beta$ 2, and prohibitin. Down-regulation of the  $\alpha$  and  $\beta$  subunits was confirmed by Western blot analysis of whole-cell extracts using G protein subunit-specific antibodies. It is noteworthy that quantitatively similar decreases in subunit abundance are observed in whole cells and the DRM fraction thereof, suggesting that the down-regulated G protein subunits are essentially confined to membrane rafts. This is consistent with the large enrichment in G $\alpha$ i and G $\beta$  subunits of the DRM fraction and the presence therein of the MOP receptor. It also indicates that the decrease in abundance of the  $\alpha$  and  $\beta$  subunits elicited by long-term morphine treatment is caused by degradation rather than relocation to another subcellular compartment, such as the nonraft fraction of the plasma membrane or the cytosol.

Morphine-induced down-regulation of the five morphine-responsive proteins proceeded in an ordered way, with down-regulation of G $\beta$  preceding that of G $\alpha$ i and prohibitin. There was clear down-regulation of G $\beta$ , but not of G $\alpha$ i or prohibitin, after 6-h exposure of the cells to the drug, a condition that

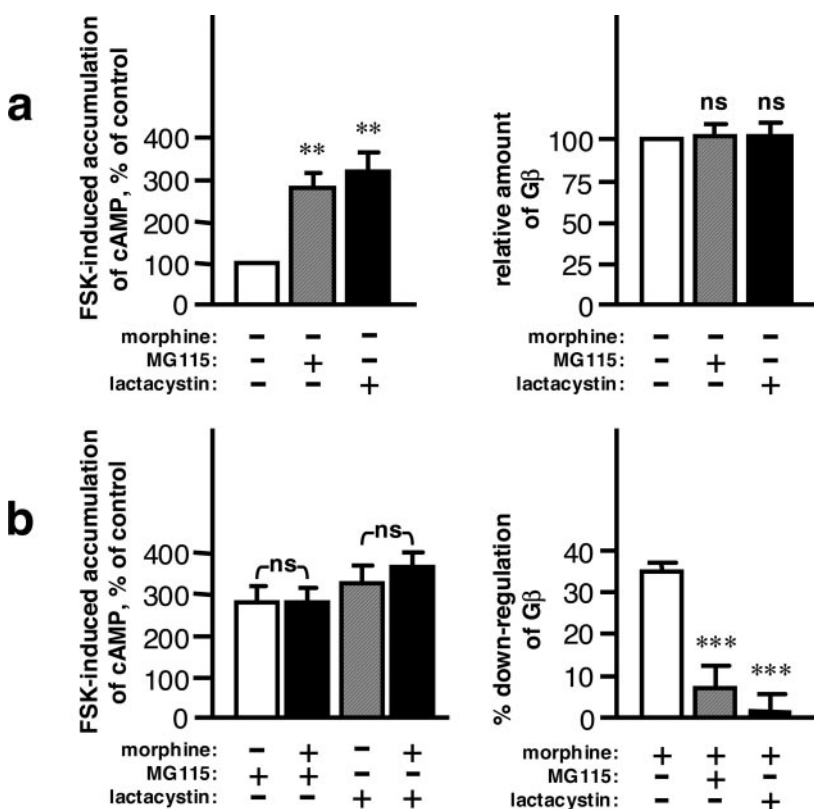


**Fig. 6.** Morphine-induced down-regulation of G $\beta$  is highly correlated with AC sensitization in SH-SY5Y sc2 cells. **a**, down-regulation of G $\beta$  elicited by long-term exposure to morphine shows a time course similar to that of AC sensitization. Cells were incubated with 1  $\mu$ M morphine for the indicated period of time and processed for Western blot quantification of G $\beta$  as described under *Materials and Methods*. Half-maximum down-regulation of G $\beta$  was attained after approximately 2 h. **b**, down-regulation of G $\beta$  elicited by long-term exposure to morphine shows a dose dependence similar to that of AC sensitization. Here, the cells were incubated for 18 h with morphine at the indicated concentration and processed for Western blot quantification of G $\beta$  as described under *Materials and Methods*. The concentration of morphine causing half-maximum down-regulation of G $\beta$  was estimated to be approximately 50 nM. Data are expressed as mean  $\pm$  S.E.M. of at least three independent experiments. **c**, correlation between AC sensitization and G $\beta$  down-regulation elicited by long-term exposure to morphine in SH-SY5Y sc2 cells. Experimental data shown in Fig. 2, **a** and **b**, were normalized to the percentage of maximum AC sensitization, and those shown in Fig. 6, **a** and **b**, were normalized to the percentage of maximum G $\beta$  down-regulation; **a** is the linear regression slope. **d**, withdrawal of morphine-conditioned SH-SY5Y sc2 cells from morphine induces G $\beta$  repletion. Drug-naïve (OMT), continually morphine-treated (CMT), and continually morphine-treated and withdrawn (CMT/W) cells were assayed for G $\beta$  content by Western blot. Long-term morphine treatment was with 1  $\mu$ M morphine for 24 h, ensuring maximum AC sensitization (Fig. 2a). CMT/W cells were assayed 6 h after removal of the drug, a condition that ensures the complete reversal of AC sensitization (Fig. 2c). In comparison with control (OMT) cells, CMT cells show a significantly diminished G $\beta$  content, whereas CMT/W cells show a G $\beta$  content not significantly different from that of control cells. Data are expressed as mean  $\pm$  S.E.M. of at least three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; ns, not significant ( $p > 0.05$ ).

caused submaximal sensitization of adenylate cyclase. This indicated a possible correlation of G $\beta$  abundance, but not G $\alpha$ i or prohibitin, with ACS. Indeed, G $\beta$  abundance and the amplitude of adenylate cyclase sensitization were found to be tightly coupled in SH-SY5Y sc2 cells. The increase in ACS with the duration of morphine exposure or with the dose of morphine was closely paralleled by a decrease in the amount of G $\beta$ . On the other hand, the decrease of AC sensitization seen after removal of the drug in morphine-pretreated cells was accompanied by an increase in the amounts of G $\beta$ . The close correlation between down-regulation of G $\beta$  and ACS in SH-SY5Y cells suggests a cause-and-effect relationship, although the precise mechanistic link remains to be determined. However, from a knowledge that AC1 and AC8 mRNA is present in neuroblastoma cells (Jang and Juhn, 2001) and that AC1 (Nielsen et al., 1996) and perhaps AC8 (Steiner et al., 2005) are inhibited by G $\beta\gamma$ , one possible mechanism whereby morphine sensitizes adenylate cyclase could be by the removal of a direct G $\beta\gamma$ -mediated inhibitory constraint. Indeed, this hypothetical mechanism may apply to the other morphine-sensitizable AC isoforms as well, because scavengers of G $\beta\gamma$  were reported previously to block morphine-induced sensitization of AC5 and AC6 (Avidor-Reiss et al., 1996; Rubenzik et al., 2001), two AC isoforms also known to be inhibited by G $\beta\gamma$  (Bayewitch et al., 1998). However, it is possible that in other cellular models, mechanisms other than down-regulation link G protein subunits with AC sensitization. For example, in MOP receptor-transfected CHO cells, long-term morphine exposure has been shown to induce changes in detergent solubility rather than abundance of G $\alpha$ i and G $\beta$ 1, correlated with the onset of AC sensitization (Bayewitch et al., 2000).

The other important observation of the present study is

that down-regulation of G $\beta$  elicited by long-term morphine treatment in SH-SY5Y sc2 cells is totally blocked by MG-115 or lactacystin. Thus, down-regulation of G $\beta$  is most probably caused by degradation by the proteasome (i.e., long-term morphine stimulates proteasomal degradation of G $\beta$  to sensitize AC in these cells). Because the  $\beta$  subunit is normally associated with a  $\gamma$  subunit to form a quasi-irreversible  $\beta\gamma$  heterodimer in the cell (Gautam et al., 1998), down-regulation of G $\beta$  is likely to reflect degradation of the entire G $\beta\gamma$  heterodimer. There is now strong evidence for the specific degradation of heterotrimeric G protein subunits by the ubiquitin-proteasome pathway, including G $\alpha$ 1 (Madura and Varshavsky, 1994; Marotti et al., 2002), G $\alpha$ o (Busconi et al., 2000), G $\alpha$ i3 (Fischer et al., 2003), and G $\beta\gamma$  (Obin et al., 2002; Hamilton et al., 2003). For instance, proteasome-dependent degradation of G $\beta$ , which we find to be down-regulated in morphine-conditioned neuroblastoma cells, was recently shown to be initiated by ubiquitylation of the  $\gamma$  subunit (Obin et al., 2002), consistent with a recent report that the  $\gamma$ 2 subunit is an N-end rule ubiquitylation substrate (Hamilton et al., 2003). It is interesting that proteasomal degradation of heterotrimeric G protein subunits seems to be dependent on accessory/adaptor regulatory proteins, such as phosducin (Obin et al., 2002), the regulator of G protein signaling RGS-GAIP (Fischer et al., 2003), and perhaps also the activator of G protein signaling 3 (AGS3), which was recently reported to slow down the degradation of G $\alpha$ i and to suppress AC sensitization elicited by prolonged stimulation of the  $\alpha$ 2-adrenergic receptor in CHO cells (Sato et al., 2004). Morphine could use analogous molecular pathways, particularly the activity-dependent ubiquitylation of G $\gamma$ , and the proteasomal degradation of the ubiquitylated G $\beta\gamma$  heterodimer to sensitize adenylate cyclase in SH-SY5Y sc2 cells.



**Fig. 7.** Proteasome inhibitors block both down-regulation of G $\beta$  and AC sensitization elicited by long-term morphine exposure in SH-SY5Y sc2 cells. **a**, in control, morphine-naïve cells, MG-115 or lactacystin alone induce enhanced forskolin-stimulated production of cAMP (left), but neither of the two proteasome inhibitors affect cellular G $\beta$  content (right). Cells were incubated with 3  $\mu$ M MG-115 or 10  $\mu$ M lactacystin for 6 h, in the absence of morphine, and assayed for forskolin-induced accumulation of cAMP or processed for Western blot G $\beta$  quantification, as described under *Materials and Methods*. **b**, in the presence of MG-115 or lactacystin, morphine did not induce further enhancement of forskolin-induced accumulation of cAMP over that produced by MG-115 or lactacystin alone in SH-SY5Y sc2 cells (left). Morphine did not induce down-regulation of G $\beta$  under these conditions (right). Cells were incubated with 1  $\mu$ M morphine in the presence of 3  $\mu$ M MG-115 or 10  $\mu$ M lactacystin for 6 h and assayed for forskolin-stimulated production of cAMP and G $\beta$  content, as described above. Data are expressed as mean  $\pm$  S.E.M. of at least three independent experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns, not significant ( $p > 0.05$ ).

It is clear that another critical issue will be to show that long-term morphine exposure acts in the same way in the brain as it is proposed here to do in neuroblastoma SH-SY5Y cells (i.e., via the proteasome). Although there is as yet no available direct evidence for the involvement of the proteasome in drug dependence, recent *in vivo* studies have established a role for regulators of G protein signaling in this process, with changes in RGS9 (Zachariou et al., 2003) or AGS3 (Bowers et al., 2004) brain levels contributing to the behavioral and neural plasticity associated with long-term administration of morphine or cocaine in rodents. These molecular adaptations, together with the emergent recognition that these accessory proteins function as drug-dependent regulators of heterotrimeric G protein processing by the UP pathway (see above), suggest a role of the proteasome in mediating the long-term effects of abused drugs. It is tempting to speculate that proteasomal degradation of G $\beta$  elicited by sustained activation of the MOP receptor by morphine in the brain contributes to "hypertrophy of the cAMP system", the hallmark of opiate dependence. So far, there is only mixed evidence for regulation of G $\beta$  levels in the brain of continually morphine-treated rats. Although a pioneer Western blot analysis failed to detect any significant down-regulation of G $\beta$  in locus coeruleus and frontal cortex (Nestler et al., 1989), a recent quantitative proteomic analysis of synaptic plasma membranes isolated from cerebral cortex has documented, among other protein changes, the down-regulation of G $\beta$  (Prokai et al., 2005).

In conclusion, the present work has suggested a plausible molecular mechanism for opiate-induced sensitization of adenylyl cyclase, a hallmark of opiate dependence, and identified the proteasome as mediating adaptations to long-term opiate exposure in cultured neuroblastoma cells *in vitro*. Further studies are needed to generalize the role of the ubiquitin-proteasome pathway in the actions of long-term morphine exposure *in vivo*.

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**Address correspondence to:** Dr. Jean-Claude Meunier, IPBS-CNRS UMR5089, 205 route de Narbonne, 31077 Toulouse cedex 04, France. E-mail: jean-claude.meunier@ipbs.fr

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