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CHRONIC MORPHINE TREATMENT ENHANCES PROTEASOME-DEPENDENT DEGRADATION OF Gβ IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS : CORRELATION WITH ONSET OF ADENYLATE CYCLASE SENSITIZATION

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Running title

Chronic Morphine-Induced Proteasomal Degradation of Gβγ

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Abbreviations

AC, adenylate cyclase; ACS, adenylate cyclase sensitization; cAMP, adenosine 3',5'-monophosphate; DRM, detergent-resistant membrane; LC-ESI, liquid chromatography-electrospray ionisation; MALDI-TOF, matrix-assisted laser desorption ionisation-time of flight; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis.

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ABSTRACT

The initial aim of the present study was to identify protein changes associated with chronic morphine treatment in a recombinant human neuroblastoma SH-SY5Y clone (sc2) stably overexpressing the human MOP receptor. In MOP receptor-overexpressing sc2 cells, acute morphine was found to be much more potent and efficacious in inhibiting forskolin-elicited production of cAMP, and chronic morphine 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 chronic 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 subunits αi_2, αi_3, β_1 and β_2, and prohibitin. Quantitative Western blot analyses of whole cell extracts showed that chronic morphine-induced down-regulation of Gβ, but not of the other proteins, is highly correlated (r^2 = 0.96) with sensitization of adenylate cyclase. Down-regulation of Gβ and adenylate cyclase sensitization elicited by chronic morphine treatment were suppressed in the presence of MG-115 or lactacystin. Thus, sustained activation of the MOP receptor by morphine in sc2 cells appears to promote proteasomal degradation of Gβ to sensitize adenylate cyclase. Together, our data suggest that chronically administered 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.

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It is widely agreed that dependence elicited by repeated drug use reflects neuronal changes that endurably alter synaptic transmission within select neural circuits in the central nervous system (Koob et al., 1998; Nestler, 2004; Kelly, 2004). These plastic changes are likely to be similar to those underlying memory formation and storage, as has been documented in simple neuronal systems, with second messengers such as in particular cAMP playing a key-role (Kandel, 2001). Indeed, one fundamental neuronal change associated with chronic 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 chronic opiate inhibition, known as adenylate cyclase sensitization (ACS) (Thomas and Hoffman, 1987; Johnston and Watts, 2003).

Chronic treatment of many cell types with drugs such as morphine that inhibit AC induce enhanced activity of the enzyme following withdrawal of the inhibitory drug (Thomas and Hoffman, 1987). The phenomenon was first reported by Sharma et al. (1975) who found that pre-treatment of hybrid glioma x neuroblastoma NG 108-15 cells with morphine for at least 12 hours led, upon removal of the drug, 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 characterised 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 μ-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 co-transfected with the MOP receptor and different AC isoenzymes, chronic

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morphine 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; Avidor-Reiss et al., 1997).

There is evidence that chronic morphine 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 chronically morphine-treated rats (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 CREB, 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 have sought to identify protein changes elicited by chronic exposure of neuroblastoma cells to morphine, and attempt to relate these changes with opiate dependence, as reflected by sensitization of adenylate cyclase. To this end, a recombinant human neuroblastoma SH-SY5Y sub-clone (sc2) stably overexpressing the human MOP receptor was used, in which a chronic morphine treatment induced a substantially higher degree of dependence, than in wild-type cells. Protein changes elicited by chronic morphine exposure were sought for by comparison of the sub-proteome of detergent-resistant membrane (DRM) raft preparations isolated from drug-naive and chronically 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 comprised of considerably fewer protein species than the whole cell proteome, thus allowing for a more quantitative analysis. Therefore, we expected that protein

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changes elicited by chronic morphine exposure in sc2 cells would be more readily detected in the raft sub-proteome than in the whole cell proteome. Indeed, quantitative proteomic analysis of DRM rafts isolated from untreated and chronically morphine-treated SH-SY5Y sc2 cells revealed chronic 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 αi_2, αi_3, β_1 and β_2, and prohibitin. Down-regulation of Gβ, but not of the other proteins, is strictly correlated with amplitude of ACS elicited by chronically-applied morphine, suggestive of a cause and effect relation. Furthermore, down-regulation of Gβ elicited by chronic 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 Gβ. 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 μ opioid receptor in pRC-CMV using lipofectamine. Stably transfected G418-resistant cells were grown in high glucose DMEM (Gibco BRL) containing 10% fetal calf serum, 50 μg/ml gentamicine (Gibco BRL), and 400 μg/ml G418 (Gibco BRL) in 5% CO_2 at 37°C.

Membrane preparation. Cells were harvested in ice-cold PBS, frozen at -70°C for 1 hr, thawed, and homogeneized in 50 mM Tris-HCl, pH 7.4 in a Potter Elvehjem tissue grinder. The nuclear pellet was discarded by centrifugation at 1,000 g for 10 min, and the total membrane (TM) fraction was collected upon centrifugation at 100,000 g for 35 min. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum

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albumin as standard.

Measurement of opiate binding sites. Opiate binding sites were assayed for in the TM fraction from neuroblastoma cell by using [^{3}H]diprenorphine (50 Ci/mmol; NEN Life Science Products). Membranes (10-60 µg protein) were incubated for 1 hr at 25°C with increasing concentrations of tritiated ligand in 0.5 ml 50 mM Tris-HCl buffer, pH 7.4. Non-specific binding was determined in the presence of 1 µM unlabeled diprenorphine. Bound radioligand was collected by filtration on glass fiber filters (GF/B; Whatman), and radioactivity counting performed in a Packard model 2100TR liquid scintillation analyzer.

Measurement of intracellular cAMP. 24-well plates were seeded with ~ 2x10^5 SH-SY5Y cells in culture medium and incubated for approx. 16 hr at 37°C. The culture medium was then replaced with fresh medium containing the various agents, morphine sulphate, (Francopia), MG115 (Z-Leu-Leu-Nva-H) or lactacystin (Sigma), or vehicle. After incubation for the desired time, the culture medium was removed and 300 µl fresh medium added containing 0.1 µM adenine and 1 µCi [^{3}H]adenine (24 Ci/mmole, Amersham) with or without morphine. After 1 hr at 37°C, the cells were rinsed 4 times with 500 µl 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, 0.5 mg/ml BSA; pH 7.4) and 200 µl of fresh KRH was added to each well. Intracellular accumulation of cAMP was initiated by the addition of 100 µl of KRH containing 5 µM forskolin (Sigma), 0.1 mM 3-isobutyl-1-methylxanthine (Sigma), 0.1 mM Ro20-1724 (Biomol Res.). After exactly 10 min at 37 °C, the reaction was stopped by the addition of 30 µl HCl 2.2 M and rapid mixing. The [^{3}H]cAMP content was determined by selective batch elution on acidic alumina columns, essentially as described by Alvarez and Daniels (1992).

Isolation of detergent-resistant membrane (DRM) fraction. Cells (~ 8x10^7 per gradient) were harvested in ice-cold PBS, frozen at -70°C for 1 hr, and resuspended in 1.7 ml

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of MBS (25 mM MES, 0,15 M NaCl) containing proteinase inhibitors (Complete Mini tablets, Roche) and 1% Triton X-100 (Sigma). After 20 min gentle agitation at 4°C, the lysate was mixed with an equal volume of ice-cold 80% sucrose in MBS buffer. 3 ml of the 40% sucrose/cell lysate were transferred to 12 ml Polyallomer centrifuge tubes (Beckman) on ice, overlaid successively with 6 ml of 30% sucrose and 2.5 ml of 5% sucrose in MBS, and centrifuged at 200,000 g for 18 hr 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 µl of each fraction using Sigma FAST p-nitrophenyl phosphate tablet sets. 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, 1 mM EDTA and recentrifuged at 100,000 g for 2.3 hr at 4°C. The DRM pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA and protein concentration determined using the Lowry assay.

Gel electrophoresis and image analysis. DRM proteins were solubilized in 1X Laemli sample buffer containing 5% β-mercaptoethanol, boiled for 5 min at 100°C, and separated by SDS-PAGE on 12% polyacrylamide gels (16 x 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, 0.1 % (w:v) brilliant blue G-250) for 24 hr. Gels were scanned using a GS-800 Calibrated Densitometer (Biorad) and analysed using Quantity One software (Biorad). Band size was calculated as the area under the intensity profile curve following background subtraction. To account for possible variation in protein load, Student 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 1X SDS-PAGE sample buffer

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containing 5% β-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-flotillin1 (Transduction Laboratories) and anti-T7 Tag (Novagen), polyclonal anti-actin (Sigma), polyclonal anti-Gαi (C-10, reactive with Gαi1, Gαi2 and Gαi3 subunits, Santa Cruz Biotechnology), and anti-Gβ (T-20, reactive with Gβ1, Gβ2, Gβ3 and Gβ4 subunits, Santa Cruz Biotechnology). After ECL revelation (Amersham), X-ray films were scanned using a GS-800 Calibrated Densitometer (Biorad). When required, blots were quantified using Quantity One software (Biorad), relative to 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 DTT in 0.1 M NH_4HCO_3 for 35 min at 56°C followed by incubation in 55 mM iodoacetamide in 0.1 M NH_4HCO_3 for 30 min at room temperature in the dark. Gel pieces were then washed with 0.1 M NH_4HCO_3 and acetonitrile, and dried in a vacuum centrifuge. Gel pieces were rehydrated in a sufficient covering volume of modified trypsin solution (12.5 ng/µl in 50 mM NH_4HCO_3; Promega) and incubated overnight at 37°C. Prior to peptide extraction, 0.5 µl 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_4HCO_3 and twice using 5% formic acid in 50% ACN. The peptide mixture was concentrated in a vacuum centrifuge to a final volume of 10 µl.

Mass spectrometry (MS) analysis and data base search. MALDI-TOF MS analyses were performed on a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer; Applied Biosystems). 0.5 µl of tryptic digest supernatant was applied on the MALDI target plate with 0.3 µl of matrix solution (α-cyano-4-hydroxycinnamic acid; 8 mg/ml in H_2O/acetonitrile/TFA, 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.

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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 (NCBI) databases, using Mascot software (Matrix Science). 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 2.1kV spray voltage. Chromatographic separation was performed onto a 75 µm ID x 15 cm PepMap C18 precolumn using a linear gradient of increasing ACN in water (5-50%) over 40min with 0.1% formic acid as ion pairing agent. MS and MS/MS data were recorded continuously with a 5 sec cycle time. Within each cycle, MS data were accumulated for 1 sec, followed by two MS/MS acquisitions of 2 sec each on the two most abundant ions. Dynamic exclusion was employed within 30 sec 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 NCBI databases with MS/MS spectra. Species were restricted to human.

Data analysis. Experimental data fitting and statistical analysis were performed using Prism (GraphPad Software). 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

Acute and chronic effects of morphine on forskolin-stimulated cAMP production in wild type and MOP receptor-overexpressing SH-SY5Y clones. Since our own preliminary studies had shown that wild type (wt), undifferentiated SH-SY5Y neuroblastoma cells responded poorly to both acute and chronic exposure to morphine, we generated

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transfected clones overexpressing the human MOP receptor.

The parent (wt) neuroblastoma SH-SY5Y clone, which expresses 0.37 ± 0.01 pmol opiate binding sites per mg membrane protein, as well as two selected genetically engineered sub-clones thereof, sc4 and sc2, which express respectively 0.60 ± 0.04 and 2.2 ± 0.3 pmol opiate binding sites per mg membrane protein, were tested for acute morphine 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 (Figure 1a). In wt cells, 10 μM morphine, the highest concentration tested, elicited less than 30% inhibition, whilst the opiate inhibited forskolin-stimulated production of cAMP completely (E_{max} = 98 ± 2 %), with half maximal inhibition being attained with 4 ± 0.6 nM morphine in sc2 cells. sc4 cells displayed intermediate response towards morphine with maximum inhibition being 88 ± 2 %, with half-maximum inhibition at 31 ± 5 nM morphine.

Similarly, chronic 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 (Figure 1b). An 18 hr exposure to 10 μM morphine was found to increase forskolin-stimulated cAMP production (1.31 ± 0.42)-fold in wt cells, (1.51 ± 0.11)-fold in sc4 cells, and (2.56 ± 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 appears to be a positive correlation between the level of expressed MOP receptor and the ability not only of acute morphine to inhibit AC activity, but also chronic morphine to induce ACS, a hallmark of opiate dependence.

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

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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 non sensitized AC activity.

Time-course, dose-dependence, and reversibility of morphine-induced ACS in SH-SY5Y sc2 cells. Since it responded maximally, notably in terms of ACS elicited by chronic morphine, the MOP receptor-overexpressing SH-SY5Y sub-clone 2 was selected for further investigation. ACS elicited by treatment of sc2 cells with 1 μM morphine was time-dependent and developed slowly (Figure 2a). Half-maximum sensitization was reached after 2.5 ± 0.4 hr. Maximum sensitization required that the cells be exposed to 1 μM morphine for at least 12 hr, and amounted to approx. 4.5-fold.

ACS elicited by treatment of sc2 cells with morphine for 18 hr was dose-related (Figure 2b), with half-maximum sensitization, in this case approx. 2.15-fold, being observed in the presence of 64 ± 18 nM morphine. This is a substantially higher concentration than that (4 ± 0.6 nM) causing half-maximum acute inhibition of forskolin-stimulated cAMP production in these cells.

Finally, ACS elicited by chronic exposure to morphine was reversible (Figure 2c), and diminished slowly upon drug removal. Challenging morphine-treated sc2 cells with forskolin at increasing time intervals following drug removal resulted in progressively decreased forskolin-stimulated production of intracellular cAMP, which returned to near control levels within approx. 6 hr. The half-life of ACS elicited by a 24 hr exposure to 1 μM morphine was estimated to be 1.1h.

Identification of chronic morphine-responsive proteins in SH-SY5Y sc2 cells. A proteomics approach, limited to the proteome of detergent-resistant membrane rafts was implemented (see Introduction), in order to identify proteins that might have undergone

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changes in abundance or location in response to chronic 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 detergent-resistant membrane (DRM) fraction. This fraction, recovered at the 30/5% sucrose interface is enriched in specific raft markers, in this case alkaline phosphatase (Figure 3a) a glycosylphosphatidylinositol-anchored protein, and flotillin (Figures 3b and 3c, left panel). It is also enriched in the Gαi and Gβ subunits of heterotrimeric G proteins (Figure 3c, middle and right panels), and contains the T7 epitope-tagged MOP receptor (Figure 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, acutely (15 min) morphine-treated and chronically (6 and 24 hr) morphine-treated sc2 cells was solubilized with sodium dodecyl sulphate (SDS), submitted to one-dimensional polyacrylamide gel electrophoresis (PAGE), and the proteins stained with colloidal Coomassie Blue. Figure 4a shows a representative one-dimensional protein map of the DRM fraction from neuroblastoma sc2 cells, comprised of at least 80 distinct protein bands.

Quantitative densitometric analyses of the stained gels indicated that acute 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 Figure 4a) were found to have been significantly down-regulated in rafts from chronically 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 nanoLC-ESI-MS/MS, as described in Experimental procedures. Band I/II was found to contain hetero-trimeric G protein subunits αi2 and αi3, bands III and IV, hetero-trimeric G protein subunits β1 and β2, respectively, and band V, prohibitin (Figure 4b). It is noteworthy that the down-regulated G protein subunits are established inhibitors of AC, especially isoform I which is

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expressed in neuroblastoma SH-SY5Y cells (see Discussion). A 6 hr pre-treatment with morphine resulted in a significant decrease in the relative amount of Gβ1 (-16.1 ± 2.7 %; p = 0.0236), but not of Gαi2, Gαi3, Gβ2 or prohibitin. A 24 hr pre-treatment with morphine caused a significant reduction in the relative amounts of the five proteins, Gαi2 (-16.8 ± 2.3 %; p = 0.0423), Gαi3 (-25.9 ± 1.6 %; p = 0.0175), Gβ1 (-24.1 ± 6.2 %; p = 0.0246), Gβ2 (-34.1 ± 5.6 %; p = 0.0148) (Figure 4c), and prohibitin (-38.4± 6.4 %; p = 0.0464).

Down-regulation of the G protein subunits elicited by chronic morphine treatment was confirmed by Western blot analysis of whole neuroblastoma cell extracts, using antibodies to Gαi and Gβ. Figure 5 shows that, in comparison with no pre-treatment, pre-treatment of the cells with morphine for 6 hr induced a significant decrease in the amount of Gβ (-28.5 ± 6.3 %, p = 0.004), but no significant change in the amount of Gαi (8.9 ± 9.5 %; p = 0.3816). Pre-treatment of the cells with morphine for 24 hr resulted in further down-regulation of Gβ (-35.6 ± 6.1 %, p = 0.0012), and substantial down-regulation of Gαi (-35.7 ± 4.9 %, p = 0.0003). Significantly, down-regulation of Gβ but not Gαi (or prohibitin), was apparent after 6 hr of exposure to morphine, a condition producing sub-maximal ACS in sc2 cells (see Figure 2a), making it clear that down-regulation of Gβ but not Gαi (or prohibitin), could be correlated to ACS in SH-SY5Y sc2 cells. The remainder of the study therefore focused specifically on Gβ.

Correlation between levels of Gβ and ACS in SH-SY5Y sc2 cells. A high degree of correlation existed between levels of Gβ, and amplitude of ACS elicited by chronic morphine treatment in SH-SY5Y sc2 cells. Quantitative Western blot analysis showed that down-regulation of Gβ closely paralleled ACS both with respect to the duration of exposure to morphine and the morphine dose. Half-maximum down-regulation of Gβ was attained after approx. 2 hr of treatment with 1 μM morphine (Figure 6a), a duration close to that (2.5 hr)

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necessary to produce half-maximum ACS (see Figure 2a). Likewise, the concentration of morphine eliciting half-maximum down-regulation of Gβ (Figure 6b) was calculated to be approx. 50 nM, similar to that (64 nM) producing half-maximum ACS (see Figure 2b) under the same conditions. Linear regression analysis of normalized experimental data shown in figures 2a, 2b, 6a and 6b, reveals the high correlation ($r^2 = 0.9619$) between ACS and down-regulation of Gβ (Figure 6c), a strong indication that morphine-elicited down-regulation of Gβ is involved in AC sensitization. Conversely, the decrease in ACS observed in morphine-treated cells after removal of the drug was paralleled by an up-regulation of Gβ, with cellular levels returning to control (untreated cells) values 6 hr after withdrawal, *i.e*. when ACS was no longer present (Figure 6d).

Effects of inhibiting proteasome activity on morphine-induced down-regulation of Gβ and ACS in SH-SY5Y sc2 cells. Since the ubiquitin-proteasome pathway provides one potential mechanism of G protein degradation (see Discussion), we tested whether blocking proteasome activity could affect chronic morphine-induced down-regulation of Gβ and ACS in SH-SY5Y sc2 cells. sc2 cells were therefore treated with MG115 or lactacystin, two inhibitors of proteasome activity, for 6 hr in the absence or presence of morphine.

Treating sc2 cells with either proteasome inhibitor alone was found to enhance forskolin-stimulated synthesis of intracellular cAMP (Figure 7a, left panel). However, treatment with proteasome inhibitor alone did not result in any significant change in Gβ abundance, as assessed by quantitative Western blot analysis of whole cell extracts (Figure 7a, right panel). Thus, in contrast to ACS induced by chronic morphine treatment, ACS elicited by the proteasome inhibitors appeared to be independent of Gβ. We verified (data not shown) that neither MG115 nor lactacystin enhanced incorporation of [^{3}H]adenine into the intracellular ATP pool (see Experimental procedures), or induced up-regulation of the AC stimulatory Gαs subunit.

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Chronic exposure of sc2 cells to morphine in the presence of MG115 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 panel). 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, whilst chronic morphine treatment induced substantial down-regulation of Gβ in the absence of proteasome inhibitors, it did not so in the presence of MG115 or lactacystin (Figure 7b, right panel). The failure of chronic morphine to induce Gβ down-regulation in MG115- or lactacystin-treated cells provides a clear indication that down-regulation of Gβ 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 chronic morphine treatment in an improved cellular model of opiate dependence, i.e. a recombinant human neuroblastoma SH-SY5Y clone (sc2) stably over-expressing the MOP receptor, the principal if not exclusive vector of acute and chronic 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 chronic morphine to induce a substantially higher degree of adenylate cyclase sensitization (ACS), a hallmark of opiate dependence, than in wild-type cells. Based on mRNA content, SH-SY5Y cells are expected to express AC1 and AC8 (Jang and Juhnn, 2001), two of the four AC isoforms previously shown to be sensitized upon chronic 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-naive and morphine-treated cells were compared has detected the significant down-regulation of five proteins, subsequently identified by mass spectrometry to be the hetero-trimeric G protein subunits αi2, αi3, β1 and β2, and prohibitin. Down-regulation of the αi and β 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 Gαi and Gβ subunits of the DRM fraction, and the presence therein of the MOP receptor. It also indicates that the decrease in abundance of the αi and β subunits elicited by chronic morphine treatment is due to degradation rather than relocation to another subcellular compartment, such as the non raft 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 Gβ preceding that of Gαi and prohibitin. Significantly, there was clear down-regulation of Gβ, but not of Gαi or prohibitin, after 6 hr exposure of the cells to the drug, a condition that caused sub-maximal sensitization of adenylate cyclase. This indicated a possible correlation of Gβ abundance, but not Gαi or prohibitin, with ACS. Indeed, Gβ abundance and the amplitude of adenylate cyclase sensitization were found to be tightly coupled in SH-SY5Y sc2 cells. The increase in ACS with duration of morphine exposure or with dose of morphine was closely paralleled by a decrease in the amount of Gβ. Conversely, the decrease of AC sensitization seen after removal of the drug in morphine-pretreated cells was accompanied by an increase in the

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amounts of Gβ. The close correlation between down-regulation of Gβ and ACS in SH-SY5Y cells suggests a cause and effect relation, 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 Juhnn, 2001), and that AC1 (Nielsen et al., 1996), and perhaps also AC8 (Steiner et al., 2005) are inhibited by Gβγ, one possible mechanism whereby morphine sensitizes adenylate cyclase could be by removal of a direct Gβγ-mediated inhibitory constraint. Indeed, this hypothetical mechanism may apply to the other morphine-sensitizable AC isoforms as well, since scavengers of Gβγ were previously reported 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 Gβγ (Bayewitch et al., 1998). It is however 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, chronic morphine exposure has been shown to induce changes in detergent-solubility rather than abundance of Gαi and Gβ1, correlated with the onset of AC sensitization (Bayewitch et al., 2000).

The other important observation of the present study is that down-regulation of Gβ elicited by chronic morphine treatment in SH-SY5Y sc2 cells is totally blocked by MG115 or lactacystin. Thus, down-regulation of Gβ is most probably due to degradation by the proteasome, i.e. chronic morphine stimulates proteasomal degradation of Gβ to sensitize AC in these cells. Since the β subunit is normally associated with a γ subunit to form a quasi-irreversible βγ heterodimer in the cell (Gautam et al., 1998), down-regulation of Gβ is likely to reflect degradation of the entire Gβγ heterodimer. There is now strong evidence for the specific degradation of heterotrimeric G protein subunits by the ubiquitin-proteasome pathway, including Gpa1 (Madura and Varshavsky, 1994; Marotti et al., 2002), Gαo (Busconi

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et al., 2000), Gαi3 (Fischer et al., 2003), and Gβγ (Obin et al., 2002; Hamilton et al., 2003). For instance, proteasome-dependent degradation of Gβ, which we find to be down-regulated in morphine-conditioned neuroblastoma cells, was recently shown to be initiated by ubiquitylation of the γ subunit (Obin et al., 2002), consistent with a recent report that the γ2 subunit is an N-end rule ubiquitylation substrate (Hamilton et al., 2003). Interestingly, proteasomal degradation of heterotrimeric G protein subunits appears 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 degradation of Gαi and to suppress AC sensitization elicited by prolonged stimulation of the α2-adrenergic receptor in CHO cells (Sato et al., 2004). Morphine could utilize analogous molecular pathways, particularly the activity-dependent ubiquitylation of Gγ, and the proteasomal degradation of the ubiquitylated Gβγ heterodimer to sensitize adenylate cyclase in SH-SY5Y sc2 cells (work in progress).

Obviously, another critical issue will be to show that chronic morphine 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. Whilst 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 chronic 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 chronic effects of abused drugs. It is tempting to speculate that proteasomal degradation of Gβγ elicited by sustained activation of

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the MOP receptor by morphine in the brain contributes to "hypertrophy of the cAMP system", the hallmark of opiate dependence. To date, there is only mixed evidence for regulation of Gβ levels in the brain of chronically morphine-treated rats. Whilst a pioneer Western blot analysis failed to detect any significant down-regulation of Gβ 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 Gβ (Prokai et al., 2005).

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

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Footnotes

LM and JN contributed equally to this work.

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Figure legends

FIGURE 1

Acute and chronic effects of morphine on forskolin-stimulated production of cAMP in human MOP receptor-overexpressing *versus* wild type neuroblastome SH-SY5Y clones. **a)** Acute morphine is far more potent and efficacious in inhibiting forskolin-induced cAMP production in SH-SY5Y sub-clones 2 (sc2), and 4 (sc4) than in parent (wt) SH-SY5Y cells. The cells were incubated for 10 min with 5 μM forskolin and morphine at the indicated concentration, and assayed for cAMP content as described in Experimental procedures. Control refers to forskolin-stimulated cAMP production in the absence of morphine. **b)** Chronic morphine induces a substantially higher degree of adenylate cyclase sensitization in SH-SY5Y sub-clones 2 (sc2), and 4 (sc4) than in parent (wt) SH-SY5Y cells. Here, the cells were incubated with 10 μM 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 acute morphine (cf. sc2 curve in a). Cells were incubated with 1 μM 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 ± S.E.M. of at least three independent experiments, with each measurement performed in triplicate.

FIGURE 2

AC sensitization elicited by chronic 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 μM morphine for the indicated period of time,

removal of the drug, and assay of forskolin-stimulated cAMP production as described in Experimental procedures. Half-maximum sensitization was attained approx. 2.5 hr after exposure to morphine. **b)** Dose-dependence of AC sensitization was determined by incubating cells for 18 hr with morphine at the indicated concentration, washing free of the drug, and assaying for forskolin-stimulated cAMP production as described in Experimental procedures. Under these conditions, the concentration of morphine that caused half-maximum sensitization was estimated to be approx. 60 nM. **c)** The reversibility of AC sensitization was determined by incubating cells for 24 hr with 1 μM morphine, drug removal, and assay of forskolin-stimulated cAMP production at the indicated time following drug removal. The AC-sensitized state waned completely within 6 hr, with half-maximum desensitization being observed after approx. 1 hr. Controls in a, b and c refer to cells not exposed to morphine. Data are expressed as the mean ± S.E.M. of at least three independent experiments with each measurement performed in triplicate.

FIGURE 3

Isolation of detergent-resistant membrane (DRM) rafts from SH-SY5Y sc2 cells. Cells were lysed in cold Triton X-100, and the lysate floated by ultracentrifugation in a discontinuous sucrose density gradient as described in Experimental procedures. Each 1-ml gradient fraction was probed for the presence of alkaline phosphatase and flotillin, established membrane rafts markers. **a)** Assay of 50 μl aliquots of each fraction for alkaline phosphatase activity indicates that the GPI-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 μl aliquot of each gradient fraction was submitted to SDS-PAGE, transferred

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to nitrocellulose, and probed for flotillin-1 using a specific antibody. **c)** In comparison with the total membrane (TM) fraction (the high speed pellet from whole cell homogenates), the DRM fraction appears to be substantially enriched in flotillin (left panel). Equal amounts (10 μg) of protein from pooled gradient fractions 3 and 4 (DRM fraction), and the total membrane (TM) fraction were submitted to SDS-PAGE, and immuno-blotted using an antibody to flotillin-1 (left panel). The TM and DRM fractions were also compared for heterotrimeric G protein subunits αi (middle panel) or Gβ (right panel) content by immunoblotting with the appropriate antibody, as described above. As is observed for flotillin, the DRM fraction is substantially enriched in Gαi and Gβ, 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 wild type (wt) cells, used as control. Immunoblotting was with an anti-T7 antibody.

FIGURE 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 in kDa) is indicated to the left. The position of the five chronic morphine-responsive (down-regulated) proteins is indicated in Roman numeral to the right. **b)** MALDI-MS and ESI-MS/MS protein identification reveals band(s) I/II to contain hetero-trimeric G protein subunits αi2 and αi3, bands III and IV, hetero-trimeric G protein subunits β1 and β2, respectively, and band V, prohibitin. MALDI peptides: number of assigned peptides with M_r(expt)-M_r(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: number of peptides for which

the spectrum allowed protein identification with Mascot individual ions scores > 28 indicating that match is statistically significant ($p < 0.05$). The accession number is that of the Swiss-Prot database. **c)** Down-regulation of Gαi2, Gαi3, Gβ1 and Gβ2 elicited by chronic morphine exposure is time-dependent. SH-SY5Y sc2 cells were exposed to 1 μM morphine for 0.25 hr (15 min), 6 hr or 24 hr, the DRM rafts isolated, and processed for densitometric quantification of the four morphine-responsive proteins as described in Experimental procedures. Note that only $G\beta_1$ is significantly down-regulated after 6 hr exposure to morphine, a condition that produces sub-maximal AC sensitization in sc2 cells (see figure 2a). Unfilled bars represent the amount of protein in DRM rafts from cells not exposed to morphine. Data are expressed as mean ± S.E.M. of at least four separate experiments. * $p < 0.05$.

FIGURE 5

Chronic morphine exposure down-regulates heterotrimeric G protein αi and β subunits in SH-SY5Y sc2 cells: validation by quantitative immunoblot analysis in whole cell extracts. **a)** SH-SY5Y sc2 cells were exposed to 1 μM morphine for 6 hr (left panel) or 24 hr (right panel), and processed for SDS-PAGE and immunoblotting using an antibody to Gαi1-3, as described in Experimental procedures. Significant down-regulation (-35.7 ± 4.9 %) of Gαi was observed after 24 hr, but not after 6 hr of treatment, similar to that observed by densitometric analysis (see figure 4c). **b)** Same as in a), except that immunoblotting was with an antibody to Gβ1-4. Significant down-regulation (-28.5 ± 6.3 %) is manifest after a 6 hr treatment with morphine, confirming the densitometric analysis (see figure 4c). 24 hr of treatment with morphine (right panel) induced 35.6 ± 6.1 % down-regulation of Gβ. Gαi and Gβ 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 ± S.E.M. of at least four separate experiments. ** $p < 0.01$, *** p

< 0.001, ns: not significant ($p > 0.05$).

FIGURE 6

Morphine-induced down-regulation of Gβ is highly correlated with AC sensitization in SH-SY5Y sc2 cells. **a)** Down-regulation of Gβ elicited by chronic exposure morphine shows a similar time course to AC sensitization. Cells were incubated with 1 μM morphine for the indicated period of time, and processed for Western blot quantification of Gβ as described in Experimental procedures. Half-maximum down-regulation of Gβ was attained after approx. 2 hr. **b)** Down-regulation of Gβ elicited by chronic exposure to morphine shows a similar dose-dependence to AC sensitization. Here, the cells were incubated for 18 h with morphine at the indicated concentration, and processed for Western blot quantification of Gβ as described in Experimental procedures. The concentration of morphine causing half-maximum down-regulation of Gβ was estimated to be approx. 50 nM. Data are expressed as mean ± S.E.M. of at least three independent experiments. **c)** Correlation beween AC sensitization and Gβ down-regulation elicited by chronic morphine exposure in SH-SY5Y sc2 cells. Experimental data shown in figures 2a and 2b were normalized to percent maximum AC sensitization, and those shown in figures 6a and 6b to percent maximum Gβ down-regulation. a is the linear regression slope. **d)** Withdrawal of morphine-conditioned SH-SY5Y sc2 cells from morphine induces Gβ repletion. Drug-naive (0MT), chronically morphine-treated (CMT), and chronically morphine-treated and withdrawn (CMT/W) cells were assayed for Gβ content by Western blot. Chronic morphine treatment was with 1 μM morphine for 24 hr, ensuring maximum AC sensitization (see figure 2a). CMT/W cells were assayed 6 hr after removal of the drug, a condition that ensures the complete reversal of AC sensitization (see figure 2c). In comparison with control (OMT) cells, CMT cells show a significantly diminished Gβ content, whilst CMT/W cells show a Gβ content not significantly different from that of control cells.

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Data are expressed as mean ± S.E.M. of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, ns: not significant ($p > 0.05$).

FIGURE 7

Proteasome inhibitors block both down-regulation of Gβ, and AC sensitization elicited by chronic morphine exposure in SH-SY5Y sc2 cells. **a)** In control, morphine-naive cells, MG115 or lactacystin alone induce enhanced forskolin-stimulated production of cAMP (left panel), but neither of the two proteasome inhibitors affect cellular Gβ content (right panel). Cells were incubated with 3 μM MG115 or 10 μM lactacystin for 6 hr, in the absence of morphine, and assayed for forskolin-induced accumulation of cAMP, or processed for Western blot Gβ quantification, as described in Experimental procedures. **b)** In the presence of MG115 or lactacystin, morphine did not induce further enhancement of forskolin-induced accumulation of cAMP over that produced by MG115 or lactacystin alone in SH-SY5Y sc2 cells (left panel). Morphine did not induce down-regulation of Gβ under these conditions (right panel). Cells were incubated with 1 μM morphine in the presence of 3 μM MG115 or 10 μM lactacystin for 6 hr, and assayed for forskolin-stimulated production of cAMP and Gβ content as described above. Data are expressed as mean ± S.E.M. of at least three independent experiments. ** $p < 0.01$, *** $p < 0.001$, ns: not significant ($p > 0.05$).

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Figure 1

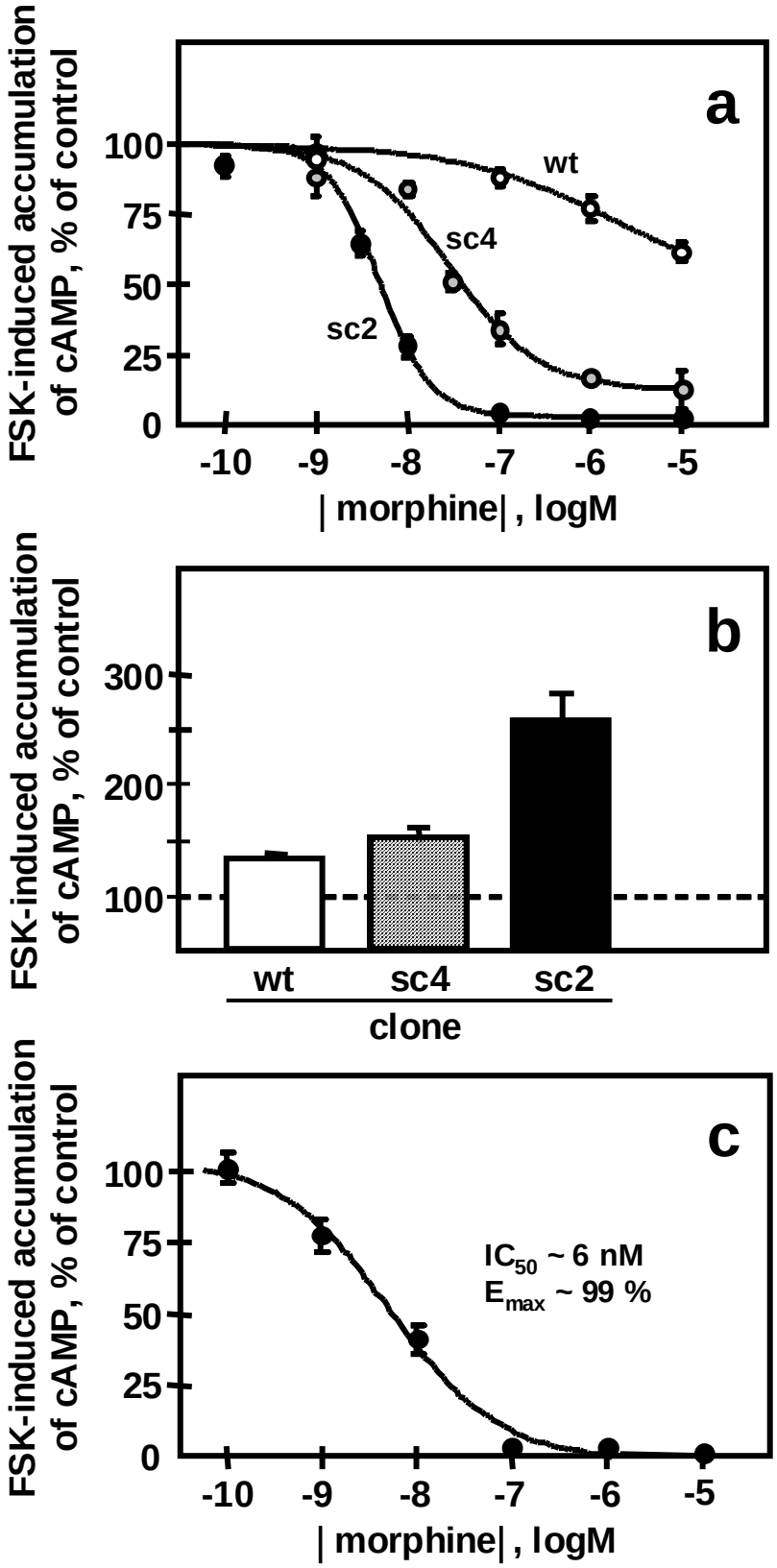

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Figure 2

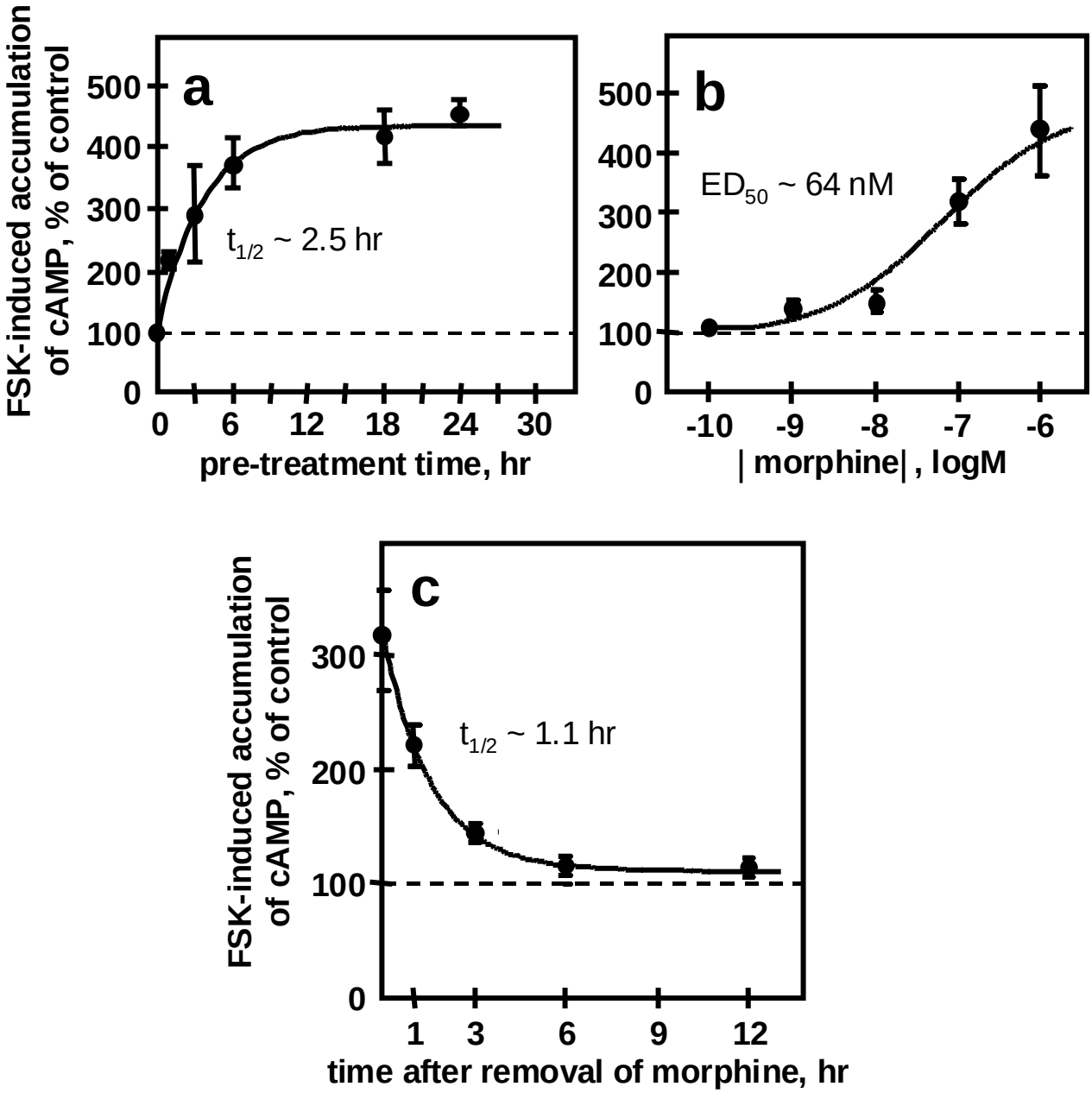

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Figure 3

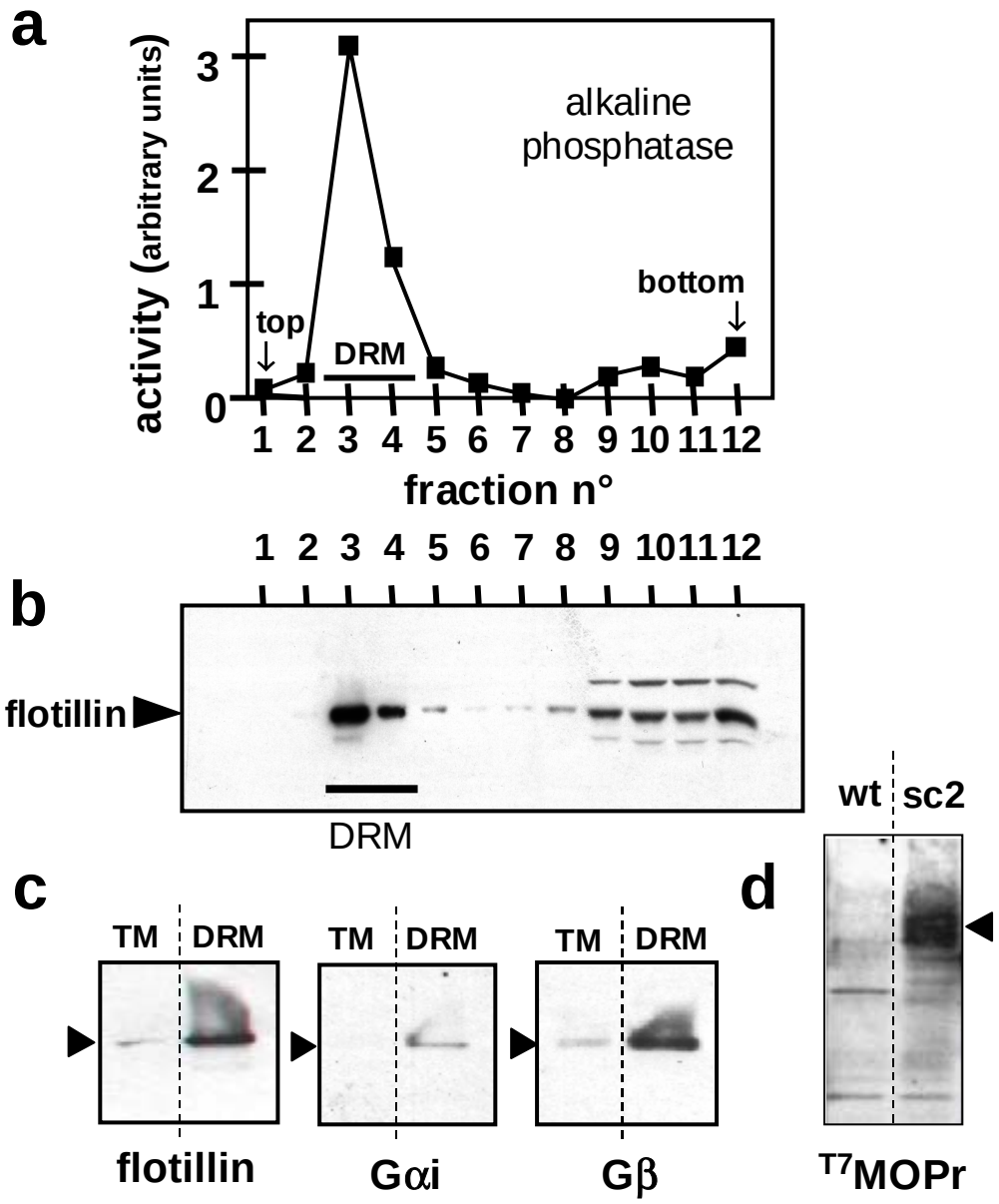

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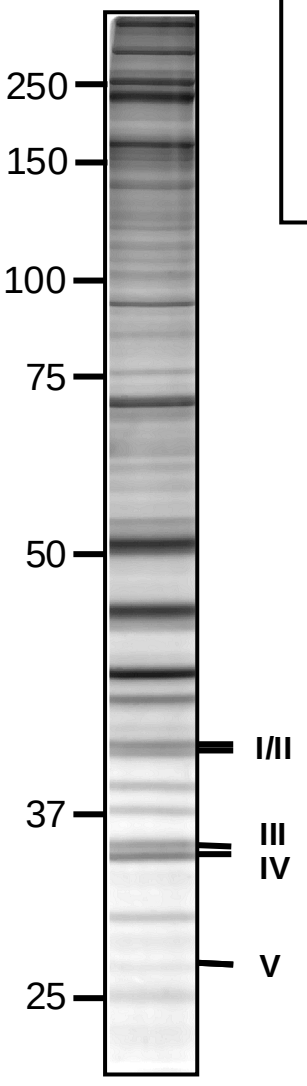
Figure 4



b

band n°	access n°	name	MW (kDa)	MALDI peptides	MS/MS peptides
I/II	P08754	**G alpha-i3**	40.5	**12** (38%)	**12**
	P04899	**G alpha-i2**	40.4	**14** (43%)	**11**
III	P04901	**G beta-1**	37.4	**9** (29%)	**8**
IV	P11016	**G beta-2**	37.3	**8** (25%)	**6**
V	P35232	**prohibitin**	29.8	**9** (31%)	**12**

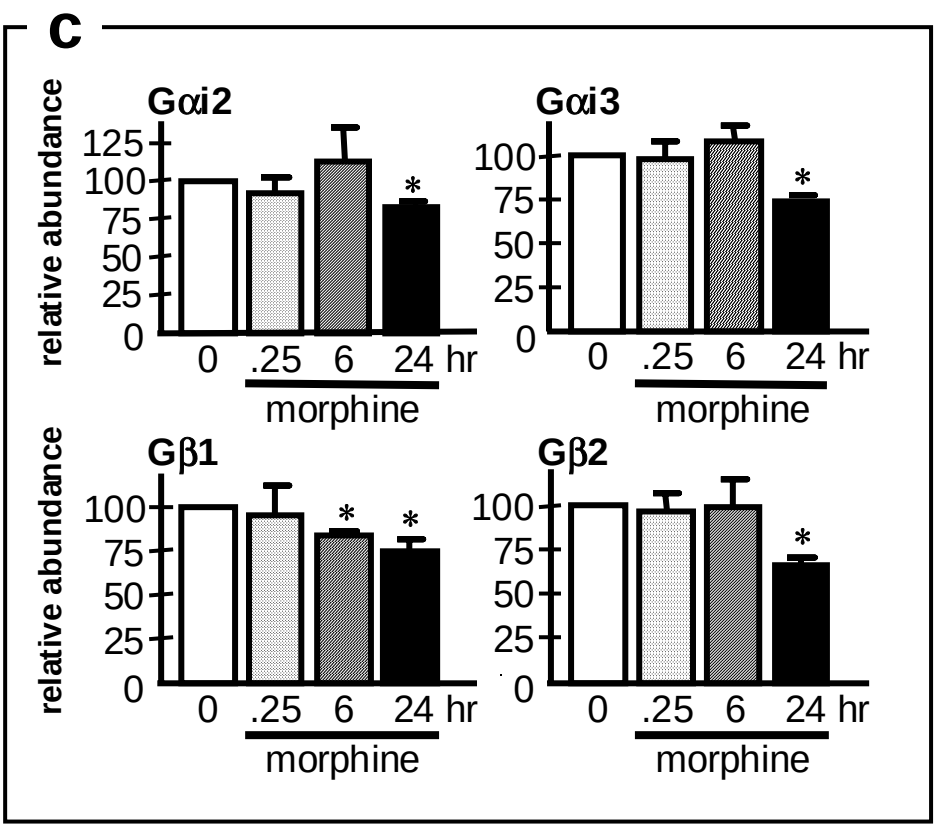


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Figure 5

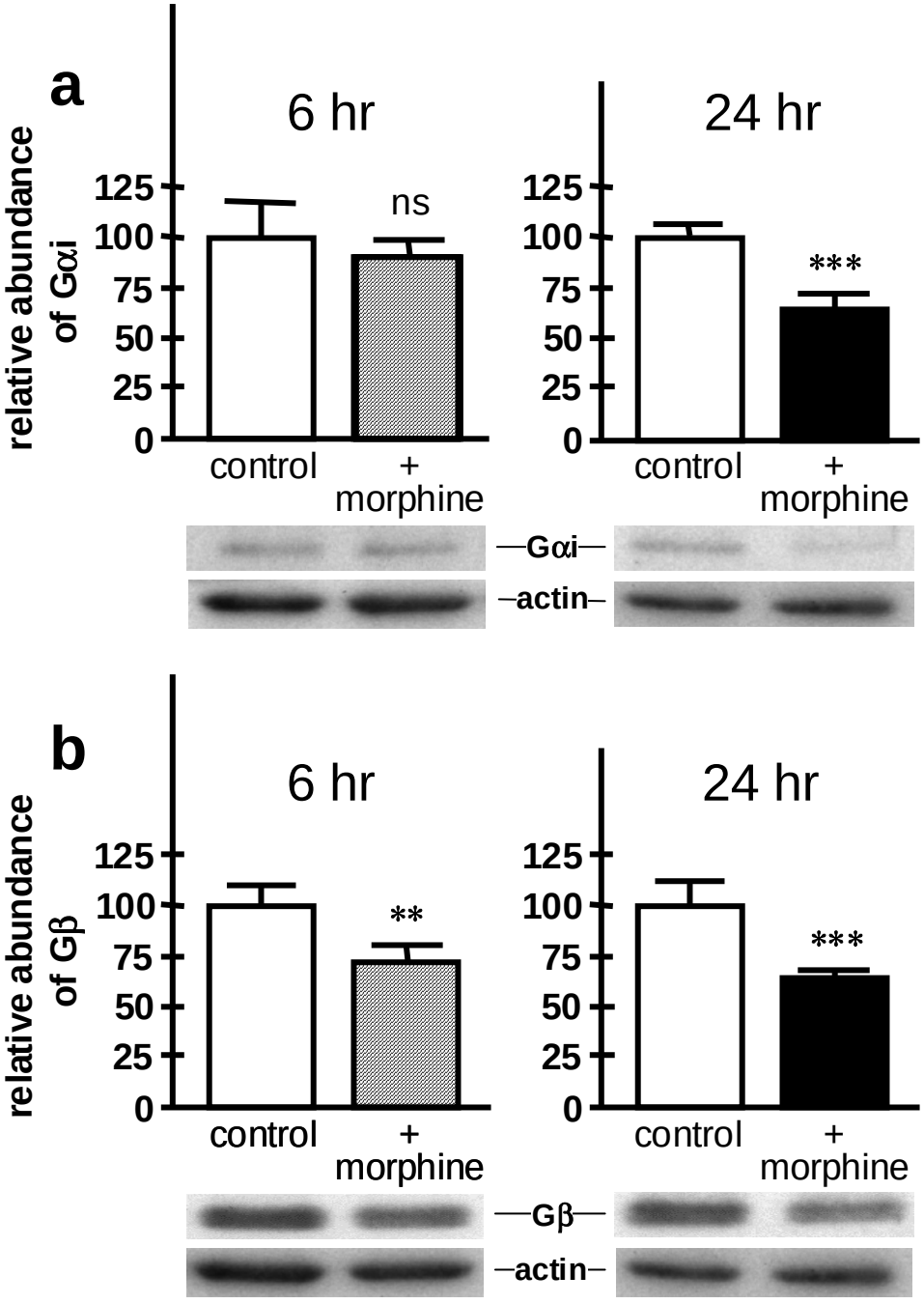

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Figure 6

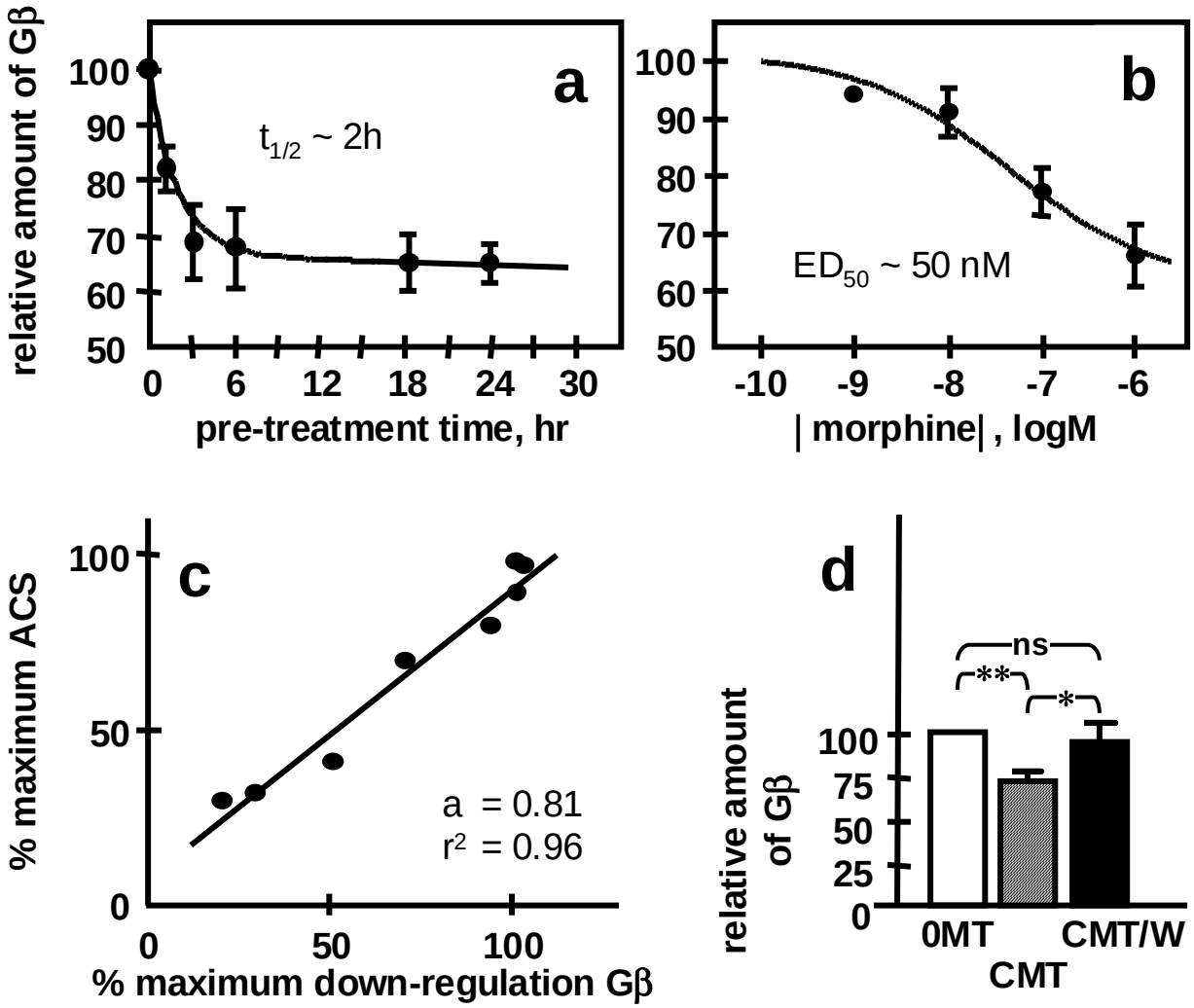

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Figure 7