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Expression of Neural RGS-R7 and Gβ5 Proteins in Response to Acute and Chronic Morphine

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The R7 subfamily of regulators of G-protein signaling (RGS) proteins (RGS6, RGS7, RGS9-2, and RGS11), and its binding protein Gβ5, are found in neural structures of mouse brain. A single intracerebroventricular priming dose of 10 nmol morphine gave rise to acute tolerance to the analgesic effects of successive identical test doses of the opioid. At 2 h after administering the acute opioid, RGS7 mRNA levels in the striatum plus those of RGS9-2 in the striatum and thalamus were increased, whereas RGS9-2 and RGS11 mRNA were reduced in the cortex. Similar but attenuated RGS-R7 mRNA changes persisted 24 h after acute morphine administration. No changes in Gβ5 mRNA levels were observed. At 2 days after commencing sustained morphine treatment, the levels of mRNA for RGS7, RGS9-2, RGS11, and Gβ5 increased in most of the brain structures studied (striatum, thalamus, periaqueductal gray matter (PAG), and cortex). In these morphine tolerant-dependent mice, the greater changes were found for RGS9-2 in the thalamus (>500%) and PAG (>200%). In post-dependent mice, the increases in RGS-R7 and Gβ5 mRNA still persisted in the PAG and striatum at 8 and 16 days after starting the chronic opioid treatment. The raised mRNA levels promoted by chronic, but not by acute, morphine, were accompanied by increases in the encoded proteins. This is probably a result of the costabilization of the RGS-R7 and Gβ5 proteins forming heterodimers. Opioid-induced adaptations of RGS-R7 and Gβ5 genes may regulate the severity of morphine-induced tolerance/dependence and the duration of the post-dependent period, helping to recover the normal response.

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INTRODUCTION

The regulators of G-protein signaling (RGS) proteins are GTPase-activating proteins (GAP) whose target are the α-subunits of the heterotrimeric Gi, Go, Gz, Gt, and Gq proteins. All contain the so-called RGS box. This is a conserved domain of approximately 130 amino-acid residues that binds the GαGDP.P transition state of activated subunits and accelerates their intrinsic GTPase activity. The mammalian RGS proteins, of which there are about 20, are grouped into five subfamilies, Rz, R4, R7, R12, and Ra, according to structural and genetic similarities (Hepler, 1999; Ross and Wilkie, 2000). *In situ* mRNA hybridization (Gold *et al*, 1997; Shuey *et al*, 1998), Northern blot analyses (Snow *et al*, 1998; Saitoh *et al*, 1999), and RT-PCR studies (Sánchez-Blázquez *et al*, 2003; Garzón *et al*, 2004) have demonstrated the presence of the mRNA in the CNS coding for the majority of the RGS and of their associated proteins.

There is increasing evidence supporting the involvement of RGS proteins in opioid effects. This has been documented in both *in vitro* (Potenza *et al*, 1999; Rahman *et al*, 1999) and *in vivo* studies (Garzón *et al*, 2001, 2003, 2004; Sánchez-Blázquez *et al*, 2003; Zachariou *et al*, 2003). The members of the RGS-R7 subfamily in CNS – RGS6, RGS7, RGS9-2, and RGS11 – mostly associate with cell membranes (Rose *et al*, 2000; Zhang and Simonds, 2000; Garzón *et al*, 2003; Sánchez-Blázquez *et al*, 2003). Their sequences contain the G-protein γ-subunit-like (GGL) domain (for a review see Hollinger and Hepler, 2002) that binds to the Gβ5 protein but not to the other Gβ subunits (Snow *et al*, 1998; Hepler, 1999; Zhang and Simonds, 2000). In nervous tissue, the RGS-R7 and Gβ5 proteins are always found as dimers, indicating this association is required for their GAP function on the corresponding GαGTP subunits (Snow *et al*, 1998; Zhang and Simonds, 2000; Sánchez-Blázquez *et al*, 2003). The RGS-R7 proteins all reduce the amplitude and duration of μ-opioid receptor-mediated effects (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003), whereas they show notable differences in their quality of action on delta agonist-mediated effects (Garzón *et al*, 2003). An observation of interest is that RGS-R7 and Gβ5 proteins

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facilitate agonist tachyphylaxis and acute tolerance at μ-opioid receptors. These regulatory processes might involve the sequestration of agonist-segregated Gα subunits by RGS-R7/Gβ5 heterodimers (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003).

The RGS genes show rapid changes in the expression following many kinds of experimental or physiological stimulus. The RGS-R4 subfamily shows remarkable plasticity. RGS4 is regulated differentially in hypothalamic nuclei by stress and glucocorticoids (Ni *et al*, 1999). It shows morphine-insensitive upregulation in a model of neuropathic pain (Garnier *et al*, 2003) and is reduced in schizophrenia (Mirnics *et al*, 2001). The acute or repeated administration of amphetamine promotes selective changes in striatal RGS-R4 mRNA levels (Burchett *et al*, 1998, 1999). Agonists and antagonists at D1 and D2 dopamine receptors also bring about changes in the level of this RGS4 mRNA (Robinet *et al*, 2001a, b; Geurts *et al*, 2002; Taymans *et al*, 2003). In rat brain, RGS-R4 and RGS-R7 genes are also responsive to acute and chronic electroconvulsive seizures (Gold *et al*, 1997, 2002). Acute morphine and cocaine decrease RGS4 in nucleus accumbens, whereas their chronic administration increases its levels (Bishop *et al*, 2002). Chronic cocaine also increases RGS9-2 in the nucleus accumbens (Rahman *et al*, 2003). Chronic opioids increase the expression of RGS4 mRNA in PC12 cells expressing opioid receptors (Nakagawa *et al*, 2001). In the rat locus coeruleus, RGS-R4 and RGS-R7 mRNA contents show no variation during chronic morphine treatment, but RGS2 and RGS4 mRNA increase after precipitating opioid withdrawal (Gold *et al*, 2003). Acute morphine increases RGS9-2 in the nucleus accumbens and the dorsal horn of the spinal cord, whereas in morphine tolerant-dependent mice this protein decreases in these structures (Zachariou *et al*, 2003).

This study examines the distribution of the mRNA and the expression levels of the RGS-R7 members present in mouse brain. Our previous studies indicate that the entire RGS-R7 subfamily plays an important role in regulating signals originated at μ-opioid receptors (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003). Therefore, we also analyzed the plasticity of the RGS-R7 members and of their partner protein Gβ5 in response to acute and chronic morphine, both in the periaqueductal gray matter (PAG) (an area involved in the analgesic effects of opioids administered by the intracerebroventricular (i.c.v.) route) and other structures of the mouse brain. The same was examined in post-dependent mice.

MATERIALS AND METHODS

Animals and Evaluation of Antinociception

Male albino mice CD-1 (Charles River) weighing 22–25 *g* were housed and used strictly in accordance with the guidelines of the European Community for the Care and Use of Laboratory Animals (Council Directive 86/609/EEC). The response of the animals to nociceptive stimuli was determined by the warm water (52°C) tail-flick test. Antinociception was expressed as a percentage of the maximum possible effect (MPE = 100 × (test latency − baseline latency)/(cutoff time {10 s} − baseline latency)).

Production of Acute Tolerance to Morphine

Animals were lightly anesthetized with ether, and 10 nmol morphine in 4 µl (priming dose) (Garzón *et al*, 2001) were i.c.v. injected into the right lateral ventricle as described previously (Sánchez-Blázquez *et al*, 1995). Controls were given saline instead. The development of acute tolerance was monitored when the priming dose had no effect on baseline latencies. Thus, 24 h later an identical dose of morphine (test dose) was i.c.v. injected into all mice of both the treatment and control groups. Acute tolerance assays were performed when the morphine reached its peak effect (after 30 min). Following the test dose, the mice were killed by decapitation at different intervals and the brain structures to be studied were obtained.

Induction and Assessment of Tolerance/Physical Dependence on Chronic Morphine Treatment

The mice were subcutaneously (s.c.) implanted with 10 ml/kg body weight of a suspension containing 50% saline (0.9% NaCl in distilled water), 42.5% mineral oil (Sigma 400-5), 7.5% mannide monooleate (Sigma M-8546), and 0.1 g/ml morphine base (adapted from Sánchez-Blázquez *et al*, 1997). The development of tolerance was monitored by measuring the analgesic response to a single i.c.v. dose of 10 nmol morphine. In mice not previously exposed to the opioid, this produced an effect of about 80% MPE in the tail-flick test. Physical dependence was evaluated by precipitating a withdrawal syndrome with 1 mg/kg of s.c. naloxone. Groups of three mice were placed in acryl-glass boxes (28 cm × 28 cm × 40 cm). The number of jumps they made were recorded over the following 10 min (Sánchez-Blázquez *et al*, 1997). The mice were killed by decapitation at different intervals after pellet implantation and brain structures were collected.

Detection of RGS-R7 and Gβ5 Proteins in Mouse Brain: Electrophoresis and Immunoblotting

The neural structures were procesed to obtain the synaptosomally enriched P2 fractions (Garzón *et al*, 2002). The brain structures required for obtaining the sought signals were pooled. Typically, three cerebral cortices and striata, five thalamus and PAG. SDS-solubilized P2 membranes were subsequently resolved by SDS/polyacrylamide gel electrophoresis (PAGE) in 8 cm × 11 cm × 1.5 cm gel slabs (7–14% total acrylamide concentration/2.6% *bis*-acrylamide crosslinker concentration). The separated proteins were then transferred onto 0.45 µm polyvinylidene difluoride membranes (Amersham Biosciences, RPN 303F). Polyclonal anti-RGS7 (SC8139), anti-RGS9 (SC8143), anti-RGS11 (SC9724) 1 : 500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-Gβ5 subunit (AB1648, Chemicon Europe Ltd, Hampshire, UK) were diluted in Tris-buffered saline–0.05% Tween-20 (TTBS) and incubated with the transfer membranes at 6°C for 12 or 24 h. The SC antibodies (raised in goat) were detected with donkey anti-goat IgG horseradish peroxidase-conjugated antiserum (Santa Cruz Biotechnology, SC2020). The Gβ5 antibody was detected with a goat anti-rabbit IgG (H + L) horseradish peroxidase-conjugated antiserum (BioRad, #170–6515).

Secondary antisera were diluted 1 : 3000 in TTBS, incubated for 2 h, and revealed with DAB substrate solution. Immunoblots were visualized with a ChemiImager IS-5500 (Alpha Innotech, San Leandro, CA) and analyzed by densitometry (AlphaEase v3.2.2). For every control neural structure and antibody used in the study, a linear relationship between the amount of protein—typically 20, 40, 60 and for some structures with weaker signals, also 80 μg protein/lane—and the intensity of the immunosignal was observed. Evaluation of the morphine-induced changes in RGS-R7/Gβ5 immunoreactivity was performed on signals inside this linear range. The data are expressed as the mean ± SEM from at least three independent determinations.

Analysis of RGS-R7 and Gβ5 mRNAs (RT-PCR)

RT-PCR is the most sensitive technique for mRNA detection and quantization currently available. The end point relative RT-PCR was used to measure changes in expression levels comparing transcript abundance across multiple samples using a coamplified internal control for sample normalization. Total RNA was harvested from mouse brain structures using a single-step procedure (Ultraspec RNA isolation system, Biotecx Laboratories, Houston, TX, USA) based on the formation of RNA complexes with guanidium molecules, followed by isopropanol precipitation. The pellet was washed in 75% ethanol, dried, resuspended in 40 μl RNA storage solution (Ambion, Austin, TX, USA), and stored at −80°C until analysis. RNA yield was determinated using UV spectrometry (260 nm). This procedure gives RNA free of DNA contamination and ready for RT-PCR without additional tratment with DNase (maker specifications). This was confirmed in routine assays with PCR performed without the previous RT step. For every neural structure analyzed, 2 μg of total RNA were reverse transcribed using the RT-PCR First Strand Synthesis Kit (RevertAid, MBI FERMENTAS) with oligo (deoxythymidine) priming as described previously (Sánchez-Blázquez *et al*, 2003). cDNA synthesis was performed at 42°C for 60 min. The template for the PCR reaction was 125 ng (RGS6), 25 ng (RGS7), 100 ng (RGS9-2 and RGS11) cDNA in a total volume of 50 μl of buffer solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 125 μM of each dNTP, 0.5 μM of each primer, and 1 U of SuperTaq thermostable DNA polymerase (Biotools, Spain).

The cDNA primers (Sigma-Genosys, Cambridge, UK) directed towards the RGS6 murine gene (AF061933) were forward 5′-GCGTTGGGGCTTCTCTTTTGATGA-3′ (sense), corresponding to base pairs 804–827 and reverse 5′-CCGCCAGCGACTTTCCCTTCTT-3′ (antisense), corresponding to base pairs 1174–1195 (designed to yield a band size of 392 bp). Those directed towards the murine gene RGS7 (AF011360) were forward 5′-CCGAGGAGGGCA GATTGAT-3′, corresponding to base pairs 123–141, and reverse 5′-GGGCAGCCATTAGCGTTCC-3′, corresponding to base pairs 420–438, in order to yield a band of 316 bp. Those towards the murine cerebral gene RGS9-2 (AF125046) were designed in a gene region that differs from the retinal gene RGS9-1 (AF011358). The secuences were: forward 5′-GTAGCCAACTTTTTCCAGAT-3′ (sense), corresponding to base pairs 2013–2032, and reverse 5′-GGTGACCCTTTTATTGTTTT-3′ (antisense), corresponding to base pairs 2413–2432 (in order to yield an expected band size of 420 bp). Those directed towards murine RGS11 (AF061934) were forward 5′-AGCGGCGGAAGGGAGACA GAAT-3′, corresponding to base pairs 440–461, and reverse 5′-CTCCTCACATGCCTCCCAGAAGC-3′, corresponding to base pairs 908–930, designed to yield a segment of 491 bp. The cDNA primers (Sigma-Genosys Ltd, Cambridge, UK) directed towards the Gβ5 murine gene (L34290) were forward 5′-GGCCGCCAAGAAGAAGT-3′ (sense), corresponding to base pairs 456–472, and reverse 5′-AGGCGC CAAATATGATGCTC-3′ (antisense), corresponding to base pairs 870–889 (designed to yield a band size of 434 bp).

Quantitative PCR amplified the RGS genes simultaneously with a sequence of the housekeeping gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that served as an internal control. The primer set directed towards the GAPDH murine gene (M32599) included 5′-ATCAC CATCTTCCAGGAGC-3′ (sense), corresponding to base pairs 260–278, and 5′-ATCACAAACATGGGGGCATCGG-3′ (antisense), corresponding to base pairs 417–438, which yielded a band of 179 bp. The GAPDH PCR products were matched with those of the RGS-R7 subfamily using GAPDH competimers (primers identical in sequence to the functional GAPDH primers but which were blocked at their 3′-end and could not, therefore, be extended by PCR). GAPDH competimers and primers were mixed at various ratios of 1 : 0.75 (RGS6), 1 : 1 (RGS7), 1 : 1.5 (RGS9-2), and 1 : 2 (RGS11). For each neural structure studied, the number of cycles was selected to give RGS-R7 amplimers in the linear phase of the PCR reaction.

In all, 31 amplification cycles were performed using thin-walled 0.2 ml PCR tubes (Ambion) in a DNA Mastercycler (Eppendorf AG, Hamburg, Germany) according to the following protocol: one cycle of 94°C (1 min) followed by 30 cycles of 94°C (20 s) and 60°C (30 s). A final 3 min incubation step was performed at 72°C. Equal volumes (15 μl) of PCR products were resolved in 2.5% agarose ethidium-bromide-stained gels with 80–1000 bp PCR markers (Biotools). The relationship between the studied RGS-R7 gene and GAPDH was calculated in terms of the relative level of expression. Fluorescent bands were visualized with UV light in a ChemiImager IS-5500 (Alpha Innotech, San Leandro, CA) and analyzed by densitometry (AlphaEase v3.2.2). The optical density of each band was compared with the respective GAPDH band after discarding the background. All experiments were repeated at least three times.

Statistical Analysis of the Results

The data collected were examined by analysis of variance (ANOVA) followed by the Student–Newman–Keuls test (SigmaStat, SPSS Science Software, Erkrath, Germany). The level of significance was set at $p < 0.05$.

RESULTS

Regional Localization of RGS-R7 Subfamily mRNA in Mouse Brain

RT-PCR assays showed amplification of the predicted sequences of RGS-R7 proteins in all the neural areas

evaluated. The data showed some region-specific patterns. Intense RGS6 mRNA signals were found in the spinal cord and pons-medulla. Weaker signals were seen for the cerebellum. RGS7 mRNA showed a more uniform distribution with highest levels in the cerebellum, followed by the hypothalamus, spinal cord, and thalamus. RGS9-2 mRNA was present in large amounts in the striatum, followed by the PAG and thalamus. The other CNS regions showed about one-third of the striatal mRNA content. RGS11 mRNA showed marked differences in distribution throughout the CNS. The strongest signals were observed in the hypothalamus and PAG, and the faintest for the striatum, cerebellum, and spinal cord (Figures 1 and 2). The Gβ5 mRNA content was similar for all CNS structures, with the exception of the hypothalamus that showed the highest levels. These results have been reported before (Sánchez-Blázquez *et al*, 2003).

Expression of RGS-R7 and of Gβ5 Genes During the Development of Acute Tolerance to Morphine

The i.c.v. injection of 10 nmol morphine produced, at its peak (30 min after injection), an analgesic effect of about 80% of the MPE allotted for this test (cutoff time of 10 s). A remnant effect of less than 20% MPE was observed 2 h after the injection (Figure 3a). After 24 h, however, no influence on the base line response of these animals could be detected. The threshold dose necessary to produce acute tolerance to morphine is about three to four times greater than that required for producing detectable analgesia (Huidobro *et al*, 1976). This interesting phenomenon appears within hours of agonist administration and lasts 2–3 days (Huidobro-Toro and Way, 1978; Garzón *et al*, 2002) (Figure 3b). The mRNA content was determined at 2 and 24 h intervals after the injection of this dose of morphine. After 2 h, RGS7 mRNA slightly but significantly increased in the striatum (about 28%), whereas that of RGS9-2 increased some 50% in this structure and some 130% in the thalamus. On the contrary, in cerebral cortex, RGS9-2 and RGS11 mRNA levels decreased by 27 and 50%, respectively. After 24 h, the changes observed at 2 h were absent or showed a tendency to diminish. No significant changes were observed for Gβ5 mRNAs levels (Figure 4).

The antibodies directed to RGS-R7 and Gβ5 proteins had been characterized previously (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003). In mouse CNS, the antibodies directed towards RGS-R7 and Gβ5 proteins bind proteins of the size expected (about 55 kDa for RGS7, 75 kDa for RGS9-2, 50 kDa for RGS11, and 39 kDa for Gβ5; Figure 5). They selectively detect the reductions brought about by the antisense oligonucleotides directed to RGS-R7/Gβ5 mRNA (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003). Moreover, the RGS-R7 antibodies proved effective in immunoprecipitating Gα subunits from CNS synaptosomal membranes and co-precipitated the Gβ5 protein that associates selectively with this subfamily of RGS proteins (Sánchez-Blázquez *et al*, 2003). Figure 5 shows representative Western blots of the cerebral cortex at 24 h post-morphine. Our data demonstrate that 24 h after administering morphine, the levels of RGS7, RGS9-2, RGS11, and Gβ5 proteins showed no significant changes

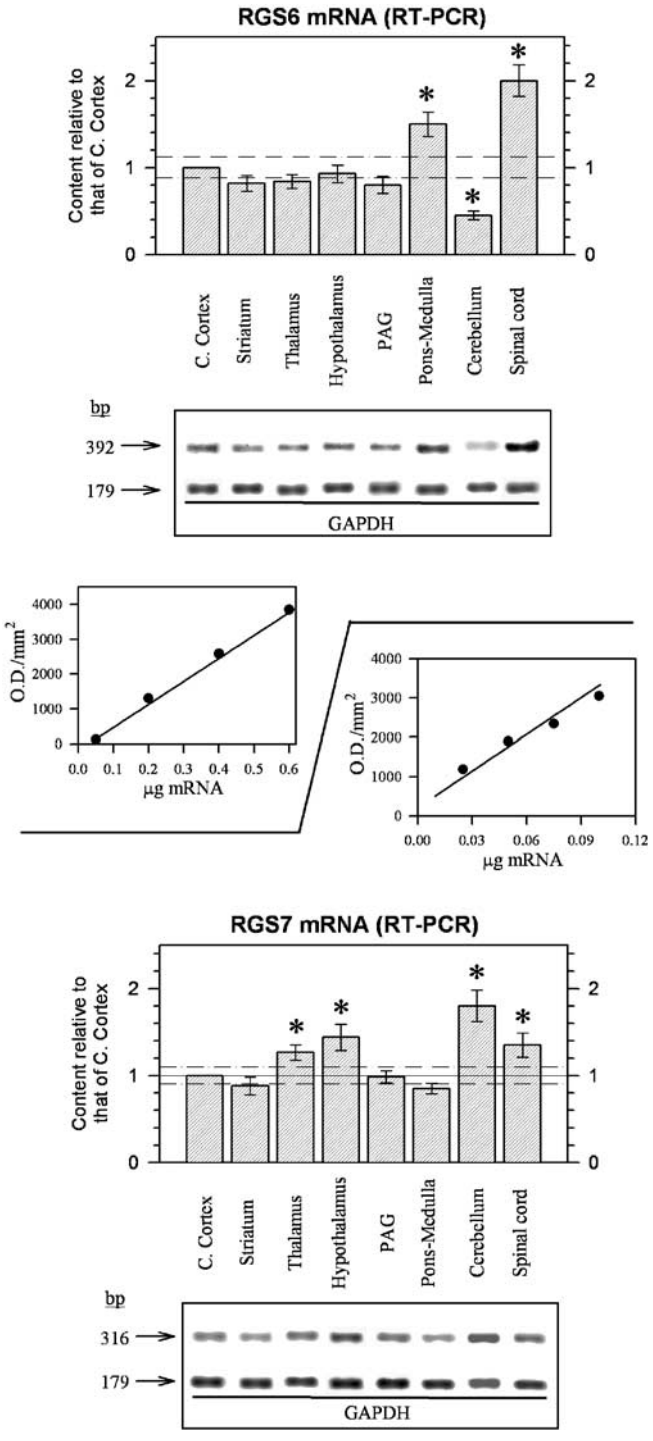

Figure 1 Detection of RGS6 and RGS7 mRNA in mouse brain. RT-PCR of RGS6 and RGS7 mRNA in different neural structures. The amplified products were 392 and 316 bp long for RGS6 and RGS7, respectively. A linear relationship was obtained for a range of micrograms of extracted RNA and the amplified 392 and 316 bp products. The signals associated to these RGS mRNA were normalized to those obtained by amplification of a segment of 179 bp GAPDH—internal standard. Each data point shown is the mean value of three determinations (each performed in triplicate) performed on pooled reverse-transcribed RNA from eight to 10 mice. *Significantly different from the mRNA levels observed for cerebral cortex—arbitrary value of 1. ANOVA followed by Student–Newman–Keuls test; $p<0.05$.

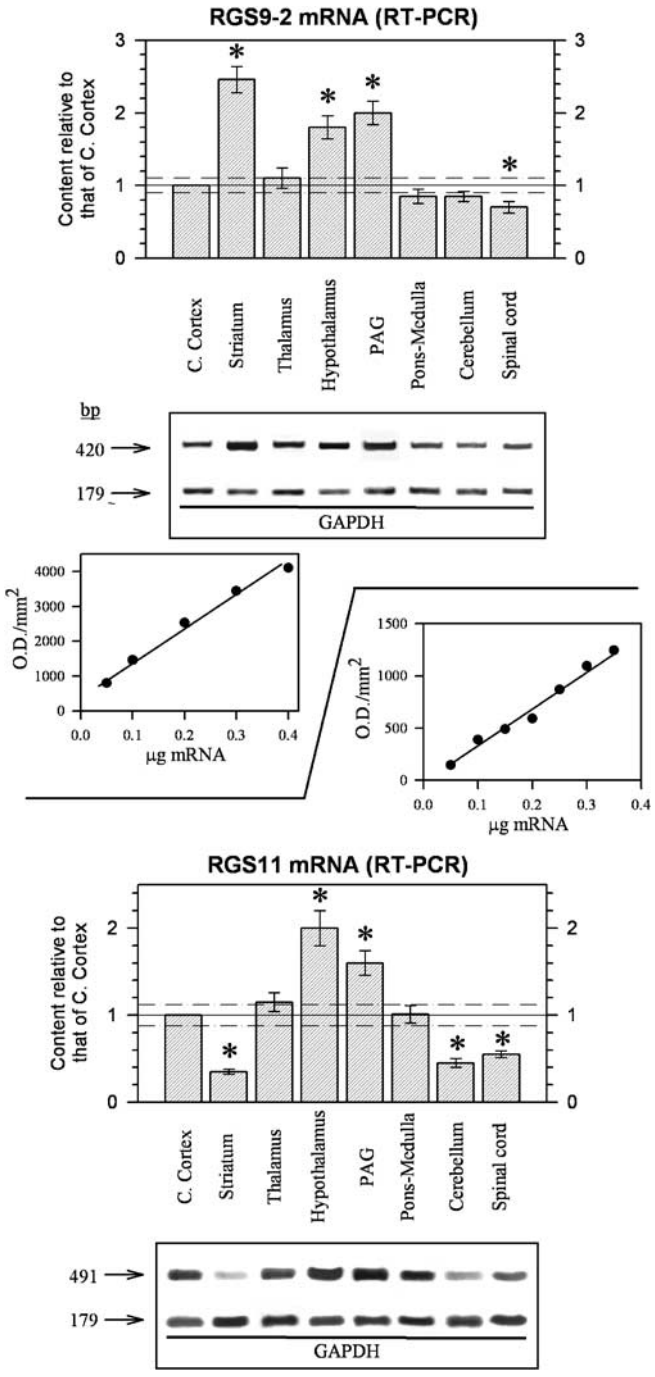

Figure 2 Detection of RGS9-2 and RGS11 mRNA in mouse brain. RT-PCR of RGS9-2 and RGS11 mRNA in different neural structures. The amplified products were 420 bp long and 491 bp for RGS9-2 and RGS11, respectively. A linear relationship was obtained for a range of micrograms of extracted RNA and the amplified products. Details as in the legend to Figure 1.

in the neural areas studied (RGS6 protein levels were not evaluated since no antibody is currently available).

RGS-R7 and Gβ5 mRNA and Protein Contents in Brain Areas of Morphine Tolerant- and Post-Dependent Mice

Mice were s.c. implanted with the oily morphine pellet. Within the first hour, the analgesic response of the mice reached the predetermined cutoff time of 10 s. Later, they developed a rapid tolerance, and this effect became almost absent at 24 h (Garzón and Sánchez-Blázquez 2001). In these animals, tolerance to the continuously delivered opioid was monitored measuring the analgesic efficacy of 10 nmol i.c.v. morphine (Figure 6). In naïve mice, this dose of the opioid produced a peak effect of about 80% of the MPE (Figure 3a). Using this protocol, it was observed that, on days 2 and 3 after pellet implantation, this dose of morphine produced modest antinociceptive effects. On the

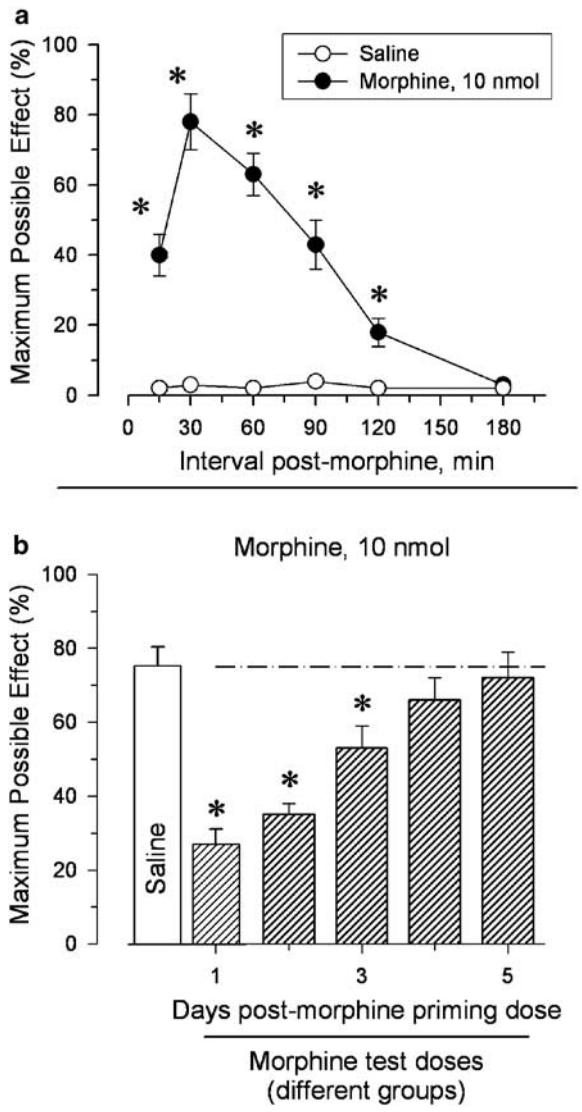

Figure 3 Morphine analgesia and induction of acute tolerance. (a) Time course of 10 nmol morphine-evoked analgesia. Mice were i.c.v. injected with morphine and analgesia determined at various intervals by the warm water 52°C tail-flick test. *Significantly different from the control group that received saline instead of the opioid. ANOVA followed by Student–Newman–Keuls test; $p<0.05$. (b) Morphine-induced acute tolerance: A priming dose of 10 nmol morphine or saline (control) was i.c.v. injected into different groups of mice. At 24 h intervals, parallel groups of morphine priming dose- and saline-treated mice were i.c.v. injected with test doses of 10 nmol morphine, and analgesia evaluated 30 min later by the tail-flick test. Values are mean ± SEM from groups of 10 to 15 mice. *Significantly different from the analgesic effect of the morphine test dose in mice that had received saline instead of the morphine priming dose (dashed line). ANOVA followed by Student–Newman–Keuls test; $p<0.05$.

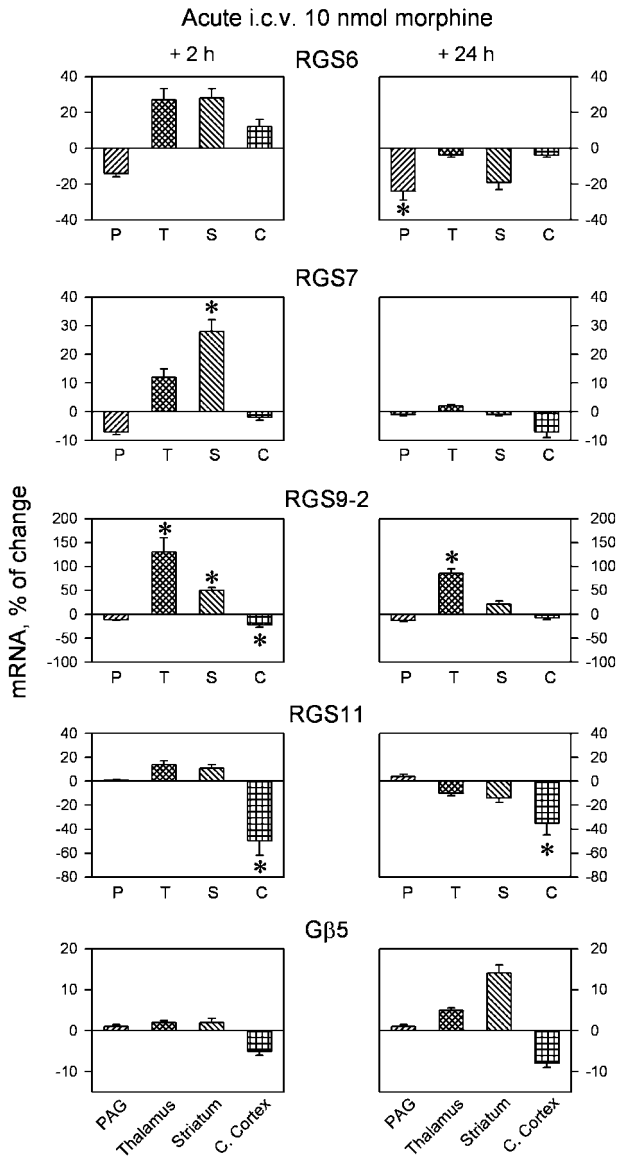

Figure 4 Influence of the development of acute tolerance to morphine on RGS-R7 and Gβ5 mRNA content in neural structures of mouse brain. The mice received i.c.v. injections of 10 nmol morphine or saline (control). At 2 or 24 h later, they were killed and the neural areas removed. The RT-PCR amplified products associated with RGS-R7 and Gβ5 mRNA were normalized with those obtained by amplification of the GAPDH internal standard. For every RGS-R7 and Gβ5, the mRNA data obtained in morphine-treated mice are expressed as a percentage of variation with respect to the results for saline-treated group (control). P (PAG), T (thalamus), S (striatum), and C (cerebral cortex). The structures of at least three mice were pooled for every determination. Each value is the mean $\pm$ SEM of three independent determinations. *Significantly different to the control group. ANOVA followed by Student–Newman–Keuls test; $p < 0.05$.

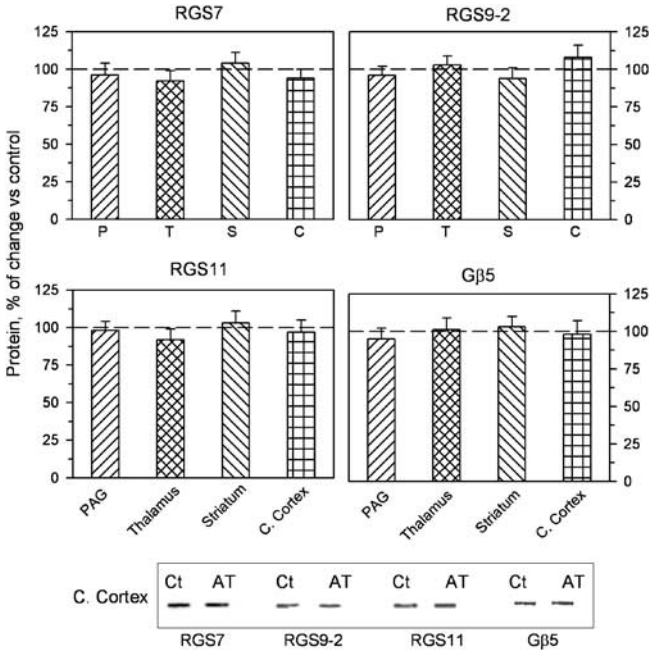

Figure 5 Influence of the development of morphine acute tolerance on RGS-R7 and Gβ5 protein content in neural structures of mouse brain. Immunodetection of RGS proteins and the partner protein Gβ5 in several CNS structures 24 h after they had received an i.c.v. injection of 10 nmol morphine. P (PAG), T (thalamus), S (striatum), and C (cerebral cortex). Each value is the mean $\pm$ SEM of three independent determinations. No significant differences were observed between the morphine-treated group and the control that received saline. Inset: representative immunosignals obtained from membranes of cerebral cortex, Ct (control group), AT (morphine acute tolerant group). Details as in the legend to Figure 4.

days that followed, its potency started to recover, and about 10 days after pellet implantation the effect of i.c.v. morphine was completely restored (Figure 6).

The jumping behavior precipitated by the opioid antagonist naloxone (1 mg/kg, s.c.) provided an index of the physical dependence on morphine. During the first 3 days after pellet implantation, the mice exhibited this withdrawal sign upon naloxone challenge. On the 4th day, its intensity started to diminish; on day 8, this behavior was greatly diminished (Figure 6). The opioid reaches its highest levels in the brain and serum during the first 24 h after pellet implantation. Later, a sustained reduction takes place, and on the 7th day morphine content is close to the detection threshold offered by the RIA procedure (Garzón and Sánchez-Blázquez, 2001). Thus, after 6 or 7 days, the opioid from the oily pellet appears to be exhausted or prevented from reaching the blood and CNS. At this stage, the morphine tolerant-dependent mice slowly recover their initial response to the opioid. They are then considered post-dependent.

During the 24 h that followed the implantation of the oily morphine pellet, RGS6, RGS7, RGS9-2, RGS11, and Gβ5 mRNA levels showed no significant changes in the neural areas analyzed. Later, the corresponding genes reacted to the chronic morphine treatment. During the post-dependent phase, RGS6 mRNA showed decreases of about 40–50% in the striatum and PAG (Figure 7). The other RGS-R7 members and Gβ5 all showed region-specific increases in their mRNA content. RGS7 mRNA increased moderately (about 30–60%) in the PAG, thalamus and cortex of tolerant-dependent mice (2 days). In the post-dependent interval (from day 8), these increases persisted in the PAG and cortex, and were also increased in the striatum (Figure 7). In tolerant-dependent mice, the brain levels of RGS9-2 mRNA greatly increased in the PAG (200%) and thalamus (500%) (Figure 8). The changes in these two structures persisted during the post-dependent period.

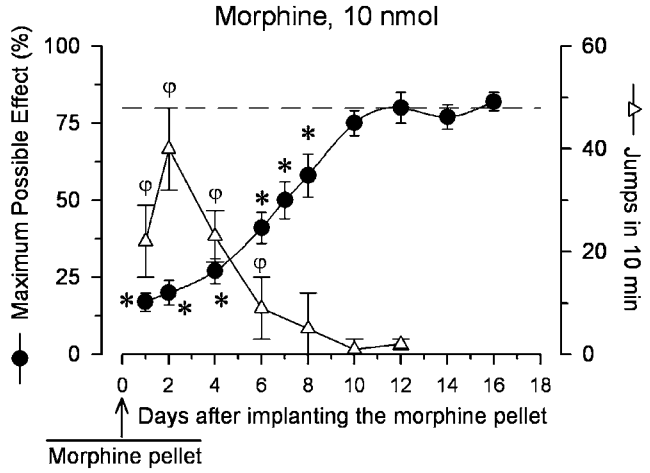

Figure 6 Development of tolerance to sustained chronic morphine. Animals s.c. injected at time zero with a 10 ml/kg body weight of a suspension containing 50% saline, 42.5% mineral oil, 7.5% mannide monooleate, and 0.1 g/ml morphine base developed rapid tolerance to the analgesia promoted by the morphine released from the suspension. The effect was absent 24 h later. The mice implanted with the morphine oily pellet were divided into several groups and the development of tolerance to morphine monitored by measuring the analgesic response 30 min after a single i.c.v. dose of 10 nmol morphine. To prevent interference caused by tolerance after receiving a single dose of the opioid, a different group of mice was used to evaluate the effect of i.c.v. morphine at every interval after implantation of the pellet. Thus, each mouse received the morphine suspension s.c. and only one i.c.v. injection of the opioid. In mice not previously exposed to the opioid, 10 nmol morphine produced an effect of about 80% MPE in the tail-flick test—indicated by the dashed line. The onset and disappearance of physical dependence on morphine was evaluated at different intervals by the jumping behavior that naloxone precipitates in tolerant-dependent mice. To precipitate the withdrawal syndrome, groups of mice implanted with the oily morphine pellet were s.c. injected with 1 mg/kg of naloxone. Jumps were recorded over the 10 min period following this challenge. The average number of jumps, mean ± SEM, is shown. Groups of 14–18 mice were used. *,φSignificantly different to control groups implanted with no oily morphine suspension. ANOVA followed by Student–Newman–Keuls test; $p < 0.05$.

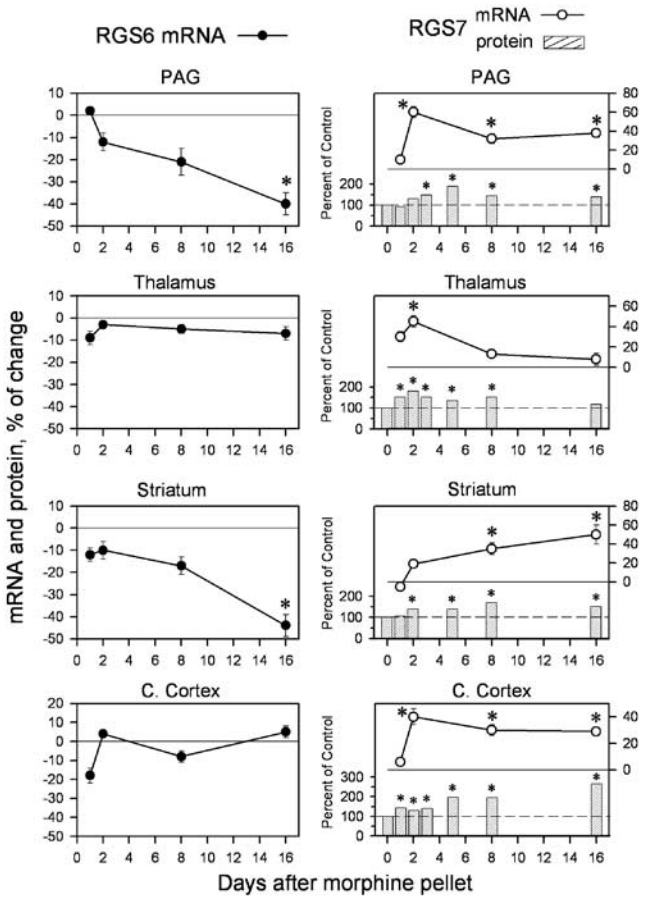

Figure 7 RGS6 and RGS7 mRNA and protein content in brain areas of morphine tolerant- and post-dependent mice. The mice s.c. implanted with the oily morphine suspension were killed at different intervals and their brain structures removed. After performing RT-PCR, the magnitudes of the amplified products, normalized with the GAPDH internal standard, were determined for every interval as the percentage variation with respect to results for the control group. Immunosignals of the expressed RGS proteins are shown at the indicated intervals postimplantation of the oily morphine pellet. The bars show the percentage variation with respect to the control group that received no chronic morphine. *Significantly different to control brain structures from mice implanted with no oily morphine suspension. ANOVA followed by Student–Newman–Keuls test; $p < 0.05$.

Increases of RGS9-2 mRNA in the striatum were also detected at these times, although no significant alteration was seen in the levels of this mRNA in the cortex. The RGS11 mRNA increased in the PAG, thalamus, striatum, and cortex. The greatest increases were of about 80–90%, and appeared during the days the animals were tolerant-dependent on morphine. During the post-dependent state, they persisted in the PAG and striatum (Figure 8). Finally, Gβ5 mRNA levels increased by 60–90% in the PAG, striatum, and cerebral cortex of morphine tolerant-dependent mice. No significant changes were observed in the thalamus. The increases in the striatum and cortex were present 8 days after initiating the morphine chronic challenge and beyond 16 days in the PAG (about 30%) (Figure 9).

Western blots revealed region-specific increases on the expression of the RGS-R7 and Gβ5 proteins (Figure 10). During the tolerance- and post-dependent phase, the levels of RGS7 protein increased moderately in the thalamus striatum and PAG (50%) and substantially in the cortex (2- to 2.5-fold) (Figure 7). In addition, although no changes were observed in the cortex, the RGS9-2 protein increased 2- to 3-fold in the PAG, striatum, and thalamus. RGS11 protein content increased about 1.5-fold in these neural structures (Figure 8). The partner Gβ5 protein increased 1.5- to 2-fold in all the brain areas studied (Figure 9). Thus, the increases in RGS-R7 and Gβ5 mRNA promoted by chronic morphine exposure reasonably paralleled the increased levels of the proteins they encode.

DISCUSSION

RT-PCR is the most sensitive technique currently available for mRNA detection and permits quantification of low levels mRNA. This approach therefore allows the detection of mRNA coding for the RGS proteins of the R7 subfamily, of the associated protein Gβ5 (Garzón *et al*, 2001; Sánchez-Blázquez *et al*, 2003; present work), and of the RGS-Rz members GAIP and RGSZ1 in several areas of the mouse brain (Garzón *et al*, 2004). The present results indicate that

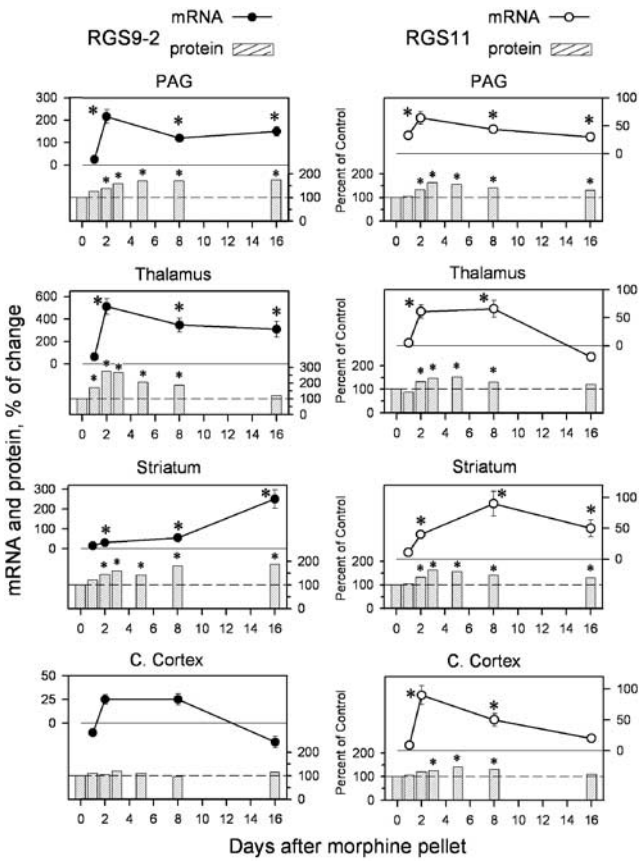

Figure 8 RGS9-2 and RGS11 mRNA and protein content in brain areas of morphine tolerant- and post-dependent mice. Experimental details as in the legend to Figure 7.

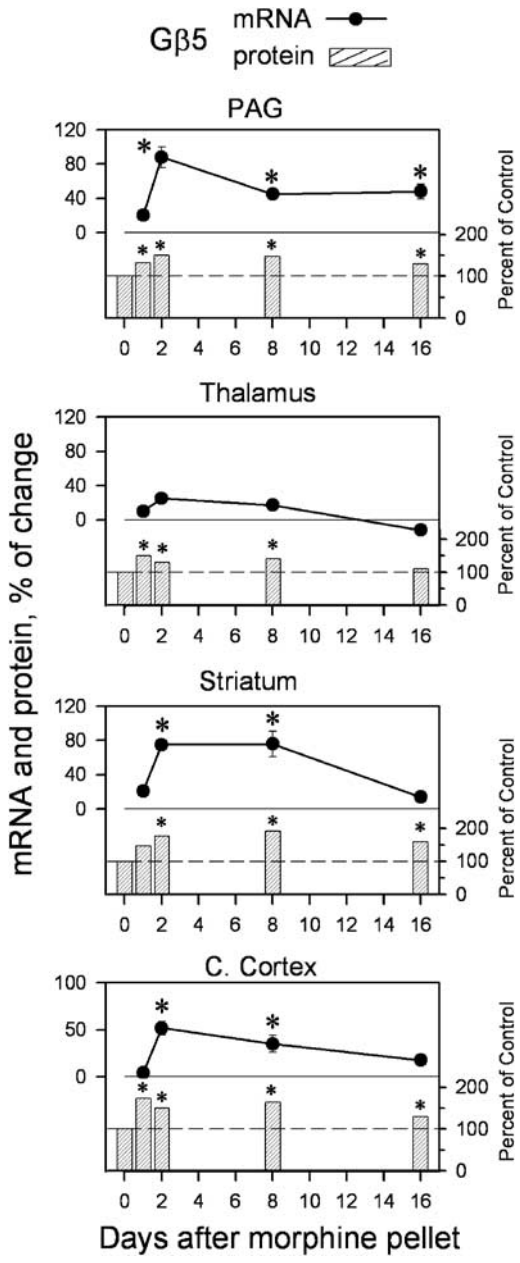

Figure 9 Gβ5 mRNA in brain areas of morphine tolerant- and post-dependent mice. Experimental details as in the legend to Figure 7.

the RGS-R7 subfamily, although widely expressed in the nervous system of the mouse, shows some region preferences. The highest levels of RGS6 mRNA were found in the spinal cord and pons-medulla, and the lowest in the cerebellum. *In situ* hybridization studies have revealed a concurrent distribution of this mRNA in rat brain, with levels in the thalamus of this species apparently higher than in the mouse (Gold *et al*, 1997). The distribution of the murine RGS7 mRNA, the highest levels of which were found in the cerebellum and spinal cord, compares satisfactorily with that reported in *in situ* hybridization studies on rat brain sections (Gold *et al*, 1997; Shuey *et al*, 1998), and also with that obtained by Northern blot analysis (Saitoh *et al*, 1999).

In the rat, RGS9-2 mRNA is highly expressed in the striatum. Lower levels are also found in the hypothalamus (Gold *et al*, 1997) and cerebral cortex (Rahman *et al*, 1999). Recently, Zachariou *et al* (2003) revealed the existence of RGS9-2 mRNA in rat PAG and spinal cord. In this species, the high levels of RGS9-2 protein expression in the striatum made its detection difficult in other areas of the CNS (Rahman *et al*, 1999; Zachariou *et al*, 2003). Using RT-PCR with mouse CNS as the substrate, we confirmed the highest levels of RGS9-2 mRNA to be in the striatum, cortex, and PAG (Garzón *et al*, 2001). The present results show that RGS9-2 mRNA is most strongly expressed in the striatum, followed by the PAG and hypothalamus. All remaining areas contained less than one-third that seen in the striatum. Therefore, compared to the rat, in the mouse the expression of RGS9-2 messenger is less restricted to the striatum. The results also show RGS11 mRNA to be unevenly distributed in the CNS, with relatively high levels in the hypothalamus and PAG, and low levels in the striatum, cerebellum, and spinal cord. Northern blot analyses show an ample distribution for RGS11 mRNA in the human brain (Snow *et al*, 1998), while in the rat, low levels and a limited distribution are reported (Gold *et al*, 1997). The mRNA of the RGS-R7-binding protein Gβ5 is reported to be present in the neural areas evaluated (Sánchez-Blázquez *et al*, 2003). The highest levels correspond to the hypothalamus, followed by those of the thalamus, PAG, pons-medulla, and cerebellum, which are all similar. The Gβ5 protein is mostly found associated with neural membranes (Watson *et al*, 1996; Betty *et al*, 1998; Sánchez-Blázquez *et al*, 2003).

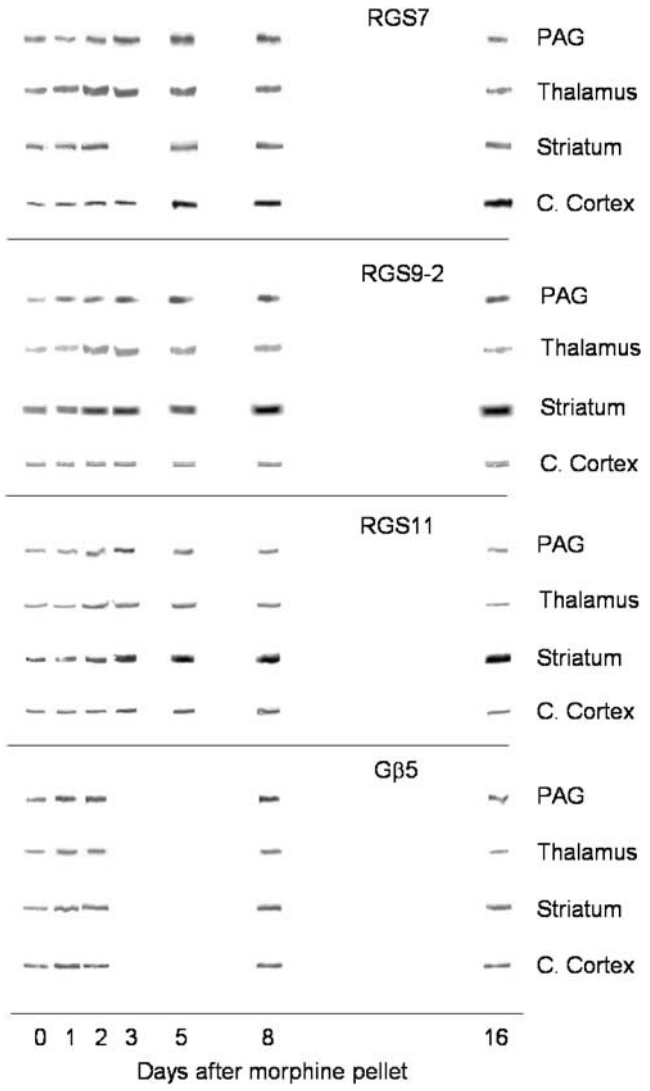

Figure 10 Expression of RGS-R7 and Gβ5 proteins in areas of mouse brain in morphine tolerant- and in post-dependent mice. The mice received a s.c. morphine oily pellet at time 0. Groups of mice were killed at the intervals indicated and immunoreactivity was monitored in synaptosomal fractions obtained from the neural areas indicated.

The expression profile observed for these RGS proteins in the mouse brain suggests that they modulate signals originating at a wide variety of G-protein-coupled receptors, including those regulating the transmission of nociceptive stimuli in PAG, thalamus, and spinal cord. In the CNS, the actions of morphine are mediated by μ-opioid receptors (Matthes *et al*, 1996). The levels of mRNA corresponding to these receptors are enriched in the thalamus, striatum, septum, cortex, and PAG. Lower levels are found in the hypothalamus, nucleus raphe, coliculli, locus coeruleus, pontine and medullary reticular nuclei, dorsal spinal horn, and the lowest in the cerebellum (Mansour *et al*, 1994; Brodsky *et al*, 1995a). Thus, the members of the RGS-R7 subfamily are present in those CNS areas where μ-opioid receptors are expressed and participate in the regulation of μ-mediated effects. In fact, it has been documented that the knockdown of RGS-R7 and Gβ5 proteins reduces opioid-induced tachyphylaxis as well as the development of acute tolerance at μ-opioid receptors (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003). This has also been observed in RGS9 knockout mice (Zachariou *et al*, 2003). The PAG plays a major role in mediating the supraspinal analgesia of opioids when given by the i.c.v. route (Yaksh *et al*, 1976). However, since PAG neurons have projections that reach other brain regions (see, for example, Wang and Wessendorf, 2002), the study of the effect of acute morphine on RGS-R7 and Gβ5 mRNA was extended to other neural structures.

An i.c.v. dose of morphine able to produce acute tolerance in mice brought about moderate changes in RGS-R7 mRNA within the first 2 h of its action. RGS6 and Gβ5 mRNAs showed no change at 2 or 24 h after acute morphine challenge. RGS7 mRNA levels increased in the striatum as did those of RGS9-2 in the striatum and thalamus. Reductions were observed in RGS9-2 and RGS11 mRNA in the cortex. These changes were much attenuated 24 h after the single dose injection of morphine. The alterations of mouse brain RGS-R7 mRNA promoted by i.c.v. acute morphine did not bring about significant changes in the expressed proteins. However, it is possible that changes occurred at the level of particular nuclei. In fact, Zachariou *et al* (2003) report that 2 h after s.c. administration of morphine, the levels of RGS9-2 protein increase in the nucleus accumbens and dorsal horn of the murine spinal cord. These areas were not included in our study with i.c.v. acute morphine. Collectively, behavioral and also the mRNA data indicate that brief to moderate activation of μ-opioid receptors triggers the endogenous pull of RGS-R7 proteins to reduce agonist effects, therefore contributing to tachyphylaxis and acute tolerance to opioids with no dramatic changes in the expression of the RGS-R7 or Gβ5 proteins. Acute morphine induced RGS-R7 mRNA changes in the striatum and cortex, areas that are not directly involved in the production of opioid antinociceptive effects via the i.c.v. route. These changes are probably induced by projections from some μ-receptor-expressing periventricular neurons that could reach these regions and activate other receptors linked to RGS-R7 regulation.

The sustained morphine treatment brought about region-specific and time-dependent changes of RGS-R7 and Gβ5 mRNA. The largest increases were those of RGS9-2 in the thalamus ($>500\%$) and PAG ($>200\%$) in the morphine tolerant-dependent mice. Smaller increases were seen in the striatum. Raised levels of RGS7, RGS11, and Gβ5 mRNA were also observed in most neural structures of these mice. In post-dependent mice, most of the RGS-R7 and Gβ5 mRNA increases persisted 8 to 16 days after starting the chronic opioid treatment. RGS6 mRNA was resistant to change during chronic morphine, but appeared reduced in the PAG and striatum of post-dependent mice. In morphine tolerant-dependent and in post-dependent mice, mRNA changes in brain structures correlated reasonably with increases in RGS-R7 proteins. Zachariou *et al* (2003) report decreases of RGS9-2 protein levels in the nucleus accumbens and spinal cord in morphine tolerant-dependent mice. These mice were s.c. administered two 25 mg hard morphine pellets on days 1 and 3, and used for Western blotting assays on day 6. It is possible that the reductions in RGS9-2 protein are promoted when morphine reaches higher concentrations in the serum and CNS, and for longer intervals than those achieved with the oily suspension. The

oily pellet provides an animal model of morphine tolerance dependence that retains the rapid rise of morphine in serum described for hard pellets, but which has the benefit of a fast decline of these levels after 4 or 5 days. Thus, their removal is not required to obtain post-dependent animals. This situation is achieved 6 to 8 days after initial delivery of the oily morphine pellet (Garzón and Sánchez-Blázquez, 2001). It should be noted that for a given neural structure, the immunodetected proteins could have originated from local RGS-R7 and Gβ5 mRNA, or have been supplied by projections from other areas of the CNS. Therefore, one should not expect to find the encoded protein restricted to high mRNA content areas. It is therefore feasible that RGS9-2 proteins originated in areas of high RGS9-2 mRNA expression, for example, the striatum and hypothalamus, are also found in the other CNS structures studied. There are many hypothalamocortical and striatocortical projections (Saper, 1985; Boylan *et al*, 1986; Hoover and Strick, 1993) that could carry these proteins to the presynaptic side of their axons. It must be remembered that RGS-R7 proteins are associated with Gβ5 and found in the cell membrane where the regulation of GαGTP subunits takes place (Rose *et al*, 2000; Zhang and Simonds, 2000; Garzón *et al*, 2003).

The increases of RGS-R7 mRNA promoted by chronic morphine, accompanied by those of Gβ5 mRNA and protein, are noteworthy. This observation agrees with the proposed coregulation of RGS-R7 and Gβ5 proteins in mammalian cells. However, this balance is not the result of transcriptional regulation. In RGS9-1 knockout mouse retina, the levels of Gβ5 long splice variant are reduced, although normal amounts of Gβ5-L mRNA are still present (Chen *et al*, 2000). Increases in RGS9-1 mRNA by transgenic expression promotes no increase of RGS9-1 protein in the retina (Chen *et al*, 2003), and Gβ5 knockout mice show reduced or zero levels of RGS-R7 proteins in the retina and striatum (Chen *et al*, 2003). Therefore, RGS-R7 protein levels are dependent on the coexpression of Gβ5 and *vice versa*. The association between RGS7 and Gβ5 to form heterodimers prevents both proteins from being degraded (Witherow *et al*, 2000). Similar observations have been made for RGS6 and Gβ5 proteins (Snow *et al*, 1999). The stabilization of these RGS-R7/Gβ5 heterodimers is critical to prevent their rapid degradation by the ubiquitin-dependent proteasome pathway. This could account for the increase of RGS-R7 and Gβ5 proteins observed as a consequence of chronic morphine. However, the reductions in cortical RGS9-2 and RGS11 mRNA observed after acute morphine brought about no reductions of the protein levels. The stabilization of the existing RGS-R7/Gβ5 complexes could delay their downregulation. In fact, treatment intervals longer than 72 h with antisense oligodeoxynucleotides are required to decrease RGS-R7 and Gβ5 protein levels in mouse brain (Sánchez-Blázquez *et al*, 2003; Garzón *et al*, 2001, 2003). Besides RGS-R7 binding to membrane proteins, post-translational modifications such as serine or threonine phosphorylation may impede or delay their degradation (Benzing *et al*, 1999, 2000; Kim *et al*, 1999). Thus, phosphorylation and/or binding to Gβ5 proteins could well preserve these RGS-R7 proteins from being rapidly degraded in the CNS.

Since RGS-R7 proteins play an essential role in the facilitation of acute tolerance at μ-opioid receptors (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003), the increases observed in RGS-R7/Gβ5 heterodimers as a consequence of morphine chronic treatment must attenuate the effects of morphine during its chronic action. Further, by reducing the sensitivity of μ receptors in post-dependent mice, the recovery of the normal function is accelerated. These morphine-induced RGS-R7 and Gβ5 alterations could also affect the function of nonopioid neurotransmitter systems. Of particular interest is the dopaminergic system, which is involved in the rewarding effects of opioids and is also intimately inter-related with RGS-R7 proteins. It is worth noting that in the CNS, acute or chronic morphine treatment produces no alteration of μ-opioid receptor mRNA levels (Brodsky *et al*, 1995b; Buzas *et al*, 1996; Castelli *et al*, 1997). However, dopamine receptor activation does lead to increased levels of μ receptor mRNA. This is observed in the nucleus accumbens after 3 days of chronic cocaine, SKF 38393, or bromocriptine (Azaryan *et al*, 1996a, b). Also, alterations in the abundance of μ receptor mRNA are observed in dopaminergically innervated brain regions accompanying amphetamine sensitization (Vecchiola *et al*, 1999; Magendzo and Bustos, 2003). Long-term haloperidol administration or irreversible blockade of D2 dopamine receptors decreases μ receptor mRNA and protein density in the striatum (Chen *et al*, 1994; Delfs *et al*, 1994; Bower *et al*, 2000). Regulation in the other direction has also been described: chronic morphine exposure and spontaneous withdrawal produce reductions in striatal D1 and D2 dopamine receptor mRNA (Georges *et al*, 1999). In μ-opioid receptor knockout mice, dopamine receptor D1/D2 mRNA expression is increased in the striatum (Park *et al*, 2001). This two-way regulation of dopaminergic D1/D2 and μ-opioid receptors, and the responsiveness of RGS-R4 and RGS-R7 mRNA levels to dopaminergic and opioidergic substances (see Introduction and present work), suggests the possibility of dopaminergic control of the facilitatory action of RGS-R7/Gβ5 complexes on μ-opioid tachyphylaxis and acute tolerance. This possibility merits consideration.

In summary, the RGS-R7 and Gβ5 genes showed responsiveness to acute and chronic administration of morphine, although for the neural areas evaluated RGS-R7 protein levels only increased when those of Gβ5 protein were raised following chronic morphine challenge. The formation of RGS-R7/Gβ5 heterodimers seems to be a requirement for the stabilization of the synthesized proteins, preventing their rapid degradation by the ubiquitin-dependent proteasome pathway. The results of this study further support the regulatory role of RGS-R7 proteins in μ-opioid receptor-mediated effects. Thus, the observed increase in RGS-R7/Gβ5 heterodimers probably helps to attenuate the regulatory pressure that morphine induces on the effectors during its chronic action. By reducing the signaling capacity of the μ-opioid receptors in post-dependent mice, the recovery of the normal responses to the endogenous opioids might be accelerated.

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