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Morphine induces short-lived changes in G-protein gene expression in rat prefrontal cortex

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Abstract

We have utilized a reverse transcriptase–polymerase chain reaction (RT-PCR) methodology followed by enzymatic restriction analysis to detect changes in G-protein mRNA levels in morphine-treated rats. The relative distribution of mRNA levels for $G\alpha_o$ $G\alpha_{i1}$, $G\alpha_{i2}$, $G\beta_1$ and $G\beta_2$ in the nucleus accumbens, striatum, locus coeruleus and prefrontal cortex was found to be similar to that previously estimated with other techniques. Morphine-induced changes of G-protein mRNA levels were detected only in the prefrontal cortex. Acute treatments (30 mg/kg, intraperitoneally) resulted in a significant increase of $G\alpha_o$ mRNA and significant decreases of $G\alpha_{i1}$ and $G\alpha_{i2}$ mRNAs. Chronic morphine administration (10–50 mg/kg over 14 days, intraperitoneally) increased $G\beta_1$ and $G\alpha_{i1}$ and $G\alpha_{i2}$ mRNAs levels to 148%, 410% and 451% of control, respectively. G-protein mRNA returned to control levels within 48 h of termination of the chronic treatments. The morphine-induced changes in G-protein mRNA levels may reflect changes in gene expression and could result in changes in G-protein levels affecting signal transduction pathways in chronically treated animals.

Keywords: Morphine; G-protein gene expression; RT-PCR (reverse transcriptase–polymerase chain reaction); Withdrawal

1. Introduction

The phenomena of tolerance and dependence to psychoactive drugs are believed to involve both transient and long-term changes of gene expression in specific brain regions (Nestler and Aghajanian, 1997). The identification of these gene expression changes is of critical importance for the understanding of addictive behaviour and, in particular, for the prevention of relapse into drug use in previously addicted individuals. The two major brain regions involved in drug addiction behaviours are the mesocorticolimbic dopamine system, which originates in the ventral tegmental area and projects to various areas including the nucleus accumbens and the prefrontal cortex (Koob, 1992), and the locus coeruleus, the main noradrenergic brain center (Nestler et al., 1994). Dopaminergic systems play a predominant role in reward and addictive behaviours but other neurotransmitters systems including noradrenergic, GABergic, glutamergic and opioid systems are also implicated (Koob, 1992; Zhang et al., 1997).

Morphine, the main pharmacologically active alkaloid of opium poppy, is a potent analgesic with known addictive properties. Morphine action is predominantly mediated by the μ-opioid receptor (Matthes et al., 1996), which is coupled to inhibitory G-proteins (Williams et al., 1988). Binding of morphine to its receptor causes the release of the G-proteins coupled to the opioid receptors and their dissociation into Gα and Gβ-γ subunits, which interact with a variety of effectors. G-proteins are widely distributed in the brain and can couple with a large family of G-protein coupled receptors. Several genes encoding G-protein subunits have been cloned (Downes and Gutman, 1999) and localized throughout the brain (Brann et al., 1987; Betty et al., 1998). In vitro studies have shown that specific G-proteins subtypes can activate different effectors, and that certain receptors can associate with more than one subtype of G-protein oligomeric complex

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(Raymond, 1995). Changes in the resting levels of G-proteins subtypes could, therefore, have an effect on intracellular signalling pathways by altering the subtype of G-protein associated with a specific receptor and, consequently, altering the type of effectors activated upon release of G-proteins (Morris and Malbon, 1999).

Several studies have shown that chronic exposure to morphine alters cAMP pathways and causes a constant elevation of adenyl cyclase, cAMP-dependent protein kinase and cAMP response element binding protein (CREB) levels (Nestler and Aghajanian, 1997). To identify the molecular mechanisms of these changes, it is necessary to establish whether gene expression of other elements of the opiate signalling pathway, namely, the opiate receptors and the G-proteins, are also altered. Chronic morphine treatment of in vitro cell cultures results in down regulation of opioid receptors (Law et al., 1984). However, the interpretation of in vivo studies of morphine-induced changes in opioid receptor expression is more complex. (Nestler, 1993; Yoburn et al., 1993; Duttaroy et al., 1999). The effect of chronic morphine on the expression of G-protein subunits has been studied both at the RNA and protein level. Chronic in vivo activation of the μ opioid receptor with the narcotic agonist fentamyl caused a significant increase of $G\alpha_i$, $G\alpha_o$ and Gβ in the guinea-pig myenteric plexus (Lang and Schulz, 1989). Several studies have reported changes in chronic morphine-induced changes of G-protein subunit levels in the central nervous system but there are some discrepancies in the specific regions where these changes have been detected. Chronic morphine lowered the level of $G\alpha_i$ in the nucleus accumbens and increased $G\alpha_i$ and $G\alpha_o$ in the amygdala and locus coeruleus without affecting other areas (Nestler et al., 1989; Terwilliger et al., 1991). In another study, chronic administration of morphine increased $G\alpha_s$ in the paraventricular nucleus of the hyppocampus, in the claustrum and in the endopiriform nucleus, while $G\alpha_{i2}$ was unchanged (Parolaro et al., 1993). In a previous study, we had detected increases of Gα and Gβ subunits in rat pineal glands following a single morphine injection (Chetsawang et al., 1999). Interestingly, G-protein activity was reduced in the locus coeruleus of rats chronically treated with morphine (Selley et al., 1997). Some of these discrepancies may be due to the regimes of morphine administration and to the assay methods utilized. However, it is important to establish the identity and location of the G-protein subunits whose mRNA expression level is altered by morphine administration because changes in G protein level could affect cellular signalling pathways and contribute to the molecular mechanisms underlying drug addiction. In this study, we have utilized a reverse transcriptase–polymerase chain reaction (RT-PCR) method to detect the level of $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$ $G\beta_1$ and $G\beta_2$ mRNA in four brain areas. We report that, in the prefrontal cortex, we have detected significant changes of G-protein subunit mRNA levels following both acute and chronic morphine administration.

2. Materials and methods

2.1. Animal maintenance and drug treatments

Spraque–Dawley male rats, obtained from the National Animal Breeding Center, Thailand, were housed with free access to food and water and maintained in a 12-h light/dark cycle (light on 8:00 AM). Initial body weights ranged between 200 and 250 g and increased to 300–350 g during chronic treatment. All animals were handled daily for at least 7 days before initiating experiments. All procedures were carried out with compliance to Mahidol University's and the European Community's guidelines for the use of experimental animals.

Morphine hydrochloride was dissolved in sterile water and injected in the rats intraperitoneally. Chronic treatment consisted of two daily injections (8:00 AM and 6:00 PM) of increasing concentrations of morphine: days 1–4, 10 mg/kg; days 5–6, 20 mg/kg; days 7–8, 30 mg/kg; days 9–10, 40 mg/kg; days 11–14, 50 mg/kg. Acute treatment consisted of the same regime of sterile water injections except for the last injection, which contained 30 mg/kg morphine. Control animals received an equal number of sterile water injections. Animals were sacrificed 2 h after the last injection except for the withdrawal group, which was sacrificed 48 h after the last injection of the chronic treatment regimen. Each group consisted of at least six animals.

2.2. Tissue dissection

Brains were immediately removed and dissected under the stereodissecting microscope in phosphate buffer saline (pH 7.4) at 4°C. Each brain was cut into four coronal sections: 2 mm frontally from the optic chiasma, at the optic chiasma and at 9 and 10 mm posterior of the optic chiasma. The prefrontal cortex was dissected from the upper frontal section of the brain. The striatum and nucleus accumbens were dissected from the slice frontal to the optic chiasma. The nucleus accumbens was identified as the clear tissue surrounding the anterior commissure and the striatum as the striated tissue located below the corpus callosum. The locus coeruleus, located bilaterally on the floor of the fourth ventricle in the anterior pons, was removed by punching it out from the slice posterior to the optic chiasma. Tissues were stored at −80°C until used. The accuracy of the dissection technique was tested by crystal-violet staining of cryosections of the above slices.

2.3. Amplification of G-protein transcripts

RNA was extracted with Trizol (BRL, Bethesda, USA), resuspended in water and its concentration estimated by optical density at $\lambda = 260$. RT-PCR was carried out in a single tube. Each 25 μl sample contained: 0.2 mM dNTPs (dATP, dCTP, dGTP and dTTP), 2 mM magnesium chlo-

ride, 25 pmol of each oligonucleotide primer, 0.75 units Taq DNA polymerase (Promega, Wisconsin, USA), 4 units avian myeloblastosis virus reverse transcriptase (Promega), 1 × buffer (50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton X100) and 0.5 μg of RNA. cDNA was synthesized by reverse transcriptase for 30 min at 42°C followed by 22 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C for 22 cycles and a final extension at 72°C for 7 min. The primers' nucleotide sequences were: forward primer Gα: TACAG CAACA CCATC CAGTC; reverse primer Gα: AAGTG GGTTT CTACG ATGCC; forward primer Gβ: CCGCT GCGTT CCTCC TGGGT; reverse primer rat Gβ: CCAGA CATTG CAGTT GAAGT; forward primer actin: CCCAG AGCAA GAGAG GCATC; reverse primer actin: CTCAG GAGGA GCAAT GATCT.

2.4. Measurement of G-protein subunits subtypes

RT-PCR products (15 μl) were digested with specific restriction enzymes to detect + subtypes of Gα and Gβ subunits. The Gα RT-PCR products were digested with *Bam*HI, *Sac*I, *Hin*PI to detect $G\alpha_o$, $G\alpha_{i1}$ and $G\alpha_{i2}$, respectively. The Gβ RT-PCR products were digested with *Ahd*I and *Kpn*I for $G\beta_1$ and $G\beta_2$, respectively. The reaction was performed in a final volume of 30 μl containing 1 × of the manufacturer's recommended restriction buffer and 10–20 units of each enzyme for 60 min at 37°C. The products were electrophoresed in a nondenaturing 8% acrylamide gel, stained with ethidium bromide and scanned with GelDoc 1000 (BioRad, Hercules, USA). The intensity of the bands was quantified on a Macintosh computer with NIH Image1.5 software (freeware, National Institutes of Health, USA). Values are presented either as arbitrary units of band intensity or as a ratio of G-protein subunit band intensity divided by the intensity of actin RT-PCR products amplified from the same RNA samples and electrophoresed in the same gel. To ensure that morphine injection has no effect on actin expression, we compared the RT-PCR products of actin mRNA and another housekeeping protein, gluteraldehyde phosphate dehydrogenase (GAPDH), amplified from equal amounts of RNA extracted from control, acutely treated and chronically treated rats. The ratio of actin and GAPDH RT-PCR products remained the same in all groups. All values are averages of at least six animals ± standard error of mean (S.E.M.). Statistical significance between groups was assessed by unpaired Student's *t*-test.

3. Results

3.1. Semi-quantification of G-protein subunits

G-protein mRNAs were amplified by RT-PCR utilizing two sets of primers complementary to conserved regions of Gα and Gβ subunits, respectively. The products were digested with restriction enzymes that uniquely cut the nucleotide sequence coding for specific subunits (Fig. 1A,B). After migration on polyacrylamide gels and ethidium bromide staining, the intensity of each band was scanned and quantified. In order to utilize RT-PCR to detect changes in specific mRNA levels, it is necessary to establish amplification parameters that yield RT-PCR products proportional to the amount of RNA present in the sample (Sirirpurkpong et al., 1997). A linear relationship between the initial amount of RNA and final RT-PCR product was observed for the $G\alpha_{i1}$ subunit following 22 amplification cycles of 0.5–2.0 μg of frontal cortex RNA (Fig. 1C,D). The same parameters were found to be suitable also for the other G-protein subunits in all tissues studied. The curve in Fig. 1D can be used only to illustrate that the amplification condition used can detect a change in G-protein mRNA and not as a standard curve to estimate the amount of G-protein mRNA present in the sample because the curve was constructed with total mRNA and not G-protein specific mRNA.

3.2. Relative distribution of G-protein

The relative distribution of $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\beta_1$ and $G\beta_2$ was measured in four brain areas. Equal amounts of total RNA from each brain region were amplified by RT-PCR and digested with specific restriction enzymes in parallel. At least six animals were used for each measurement. It is important to emphasize that the methodology used does not yield absolute values of G-protein mRNA levels nor does it accurately estimate the ratio of different G-protein subunits mRNAs within the same tissue because the amplification efficiency for the different sequences may be different. The semi-quantitative RT-PCR was here used to determine the relative profiles of the G-protein subunit mRNAs among different tissues. The results (Fig. 2) indicate that the profiles of G-proteins mRNA expression varied among different tissues. For example, in the prefrontal cortex amplification of $G\alpha_{i1}$ and $G\alpha_{i2}$ mRNAs yielded similar levels of RT-PCR products, while in the striatum $G\alpha_{i2}$ RT-PCR products were about three times higher than those of $G\alpha_{i1}$. $G\alpha_{i2}$ mRNA was not detected in the locus coeruleus and $G\beta_1$ mRNA was only detected in the prefrontal cortex where it yielded similar levels of RT-PCR products to those of $G\beta_2$.

3.3. Effect of morphine on G-protein expression

The effect of morphine injection on the expression of G-protein subunits was measured in the striatum, nucleus accumbens, locus coeruleus and prefrontal cortex. Rats were treated once with 30 mg/kg morphine (acute group) or for 15 days with increasing morphine doses (10–50 mg/kg) and sacrificed 2 h (acute and chronic groups) or 48 h (withdrawal group) after the last injection. Morphine treatment induced significant changes in G-protein mRNAs only in the prefrontal cortex (Fig. 3); no significant

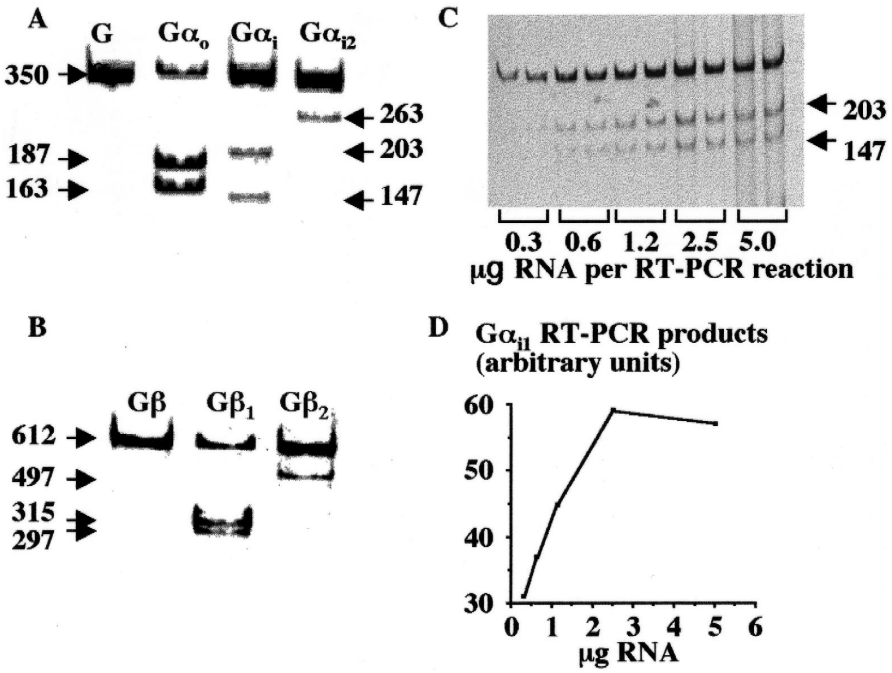

Fig. 1. Detection of G-protein subunits mRNA by RT-PCR followed by enzymatic digestion. Panels A–C are digital images of ethidium bromide stained nondenaturing acrylamide gels. Panel A: rat prefrontal cortex mRNA was amplified with Gα primers. RT-PCR products were either undigested (Gα) or digested with *Bam*HI ($G\alpha_o$), *Sac*I ($G\alpha_{i1}$), *Hin*PI ($G\alpha_{i2}$). The expected base pair lengths of the restriction digestion products are indicated. The second fragment of the $G\alpha_{i2}$ was too short to be detected by this system. Panel B: rat prefrontal cortex mRNA was amplified with Gβ primers. RT-PCR products were either undigested (Gβ) or digested with *Ahd*I ($G\beta_1$) or *Kpn*I ($G\beta_2$). The expected base pair lengths of the restriction digestion products are indicated. The second fragment of the $G\beta_2$ was too short to be detected by this system. Panel C: increasing amounts of prefrontal cortex were amplified by RT-PCR and digested with *Sac*I for the detection of $G\alpha_{i1}$ message. Panel D: quantification of the $G\alpha_{i1}$ RT-PCR digestion product (203 base pair) shown in panel C.

changes were detected in the other three brain regions studied. Acute treatment caused an increase of $G\alpha_o$ and a decrease of $G\alpha_{i1}$ and $G\alpha_{i2}$ and no changes in the Gβ subunits. Chronic administration of morphine caused an

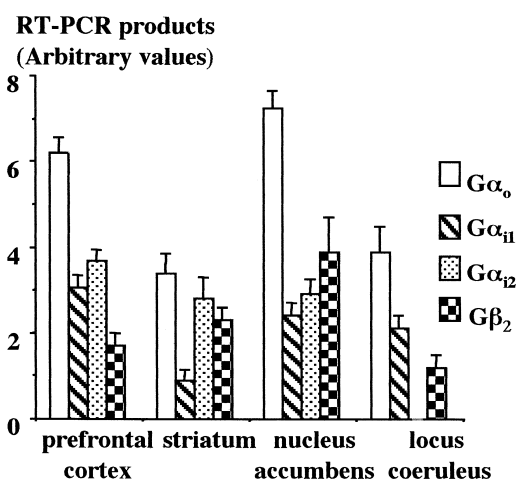

Fig. 2. Relative distribution of G-protein subunits in different brain regions. RNA from each indicated tissue was amplified with either Gα or Gβ primers and digested with restriction enzymes to detect individual subunits mRNA. Products were electrophoresed, scanned, quantified as arbitrary units of G-protein RT-PCR products and averaged. Vertical lines indicate S.E.M., $n = 6$.

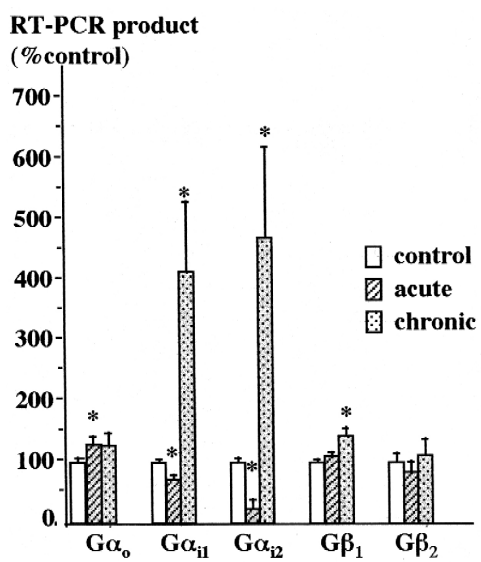

Fig. 3. Effect of morphine administration on G-protein subunit mRNA expression in prefrontal cortex. Prefrontal cortex mRNA was extracted from control, acutely and chronically morphine-treated rats. G-protein subunits RT-PCR products were quantified as a ratio of G-protein RT-PCR products divided by actin RT-PCR products and expressed as percentage of the values of control animals. Vertical lines indicate S.E.M., $n = 6$. S.E.M. of the control values indicates variation among the control samples. Asterix indicates significant difference ($p < 0.05$, Student's *t*-test) from control value.

increase in the $G\beta_1$, $G\alpha_{i1}$ and $G\alpha_{i2}$ mRNA levels; the percentage increase of the latter two were 410% and 450%, respectively. The percentage increase is greater than the linear range shown in Fig. 1D. This is because Fig. 1D was constructed by increasing total RNA, which causes the RT-PCR reaction to plateau earlier than by just increasing one RNA specie. Following 48 h of morphine withdrawal, all G-protein subunits mRNAs returned to levels that were not significantly different from control levels.

4. Discussion

In this study, we have employed a combination of RT-PCR and enzymatic restriction digests to measure G-protein subunits mRNA levels. This methodology is particularly suited for the study of the expression of genes with homologous sequences as it exploits both the high sensitivity of RT-PCR and the specificity of restriction enzymes (Yarowsky et al., 1991). We have shown previously (Sirirpurkpong et al., 1997; Nudmamud et al., 2000) and in this study that amplification conditions can be designed to obtain a linear relationship between initial amount of RNA and specific RT-PCR products thereby enabling semi-quantitative measurements of specific mRNA species.

Relative mRNA levels for G-protein subunits were measured in four brain areas. The results obtained are qualitatively similar to those obtained by others with different techniques (Brann et al., 1987, Largent et al., 1988; Parolaro et al., 1993; Betty et al., 1998) and further illustrate that different brain regions specifically regulate their basal mRNA levels of G-protein subunits. This suggests that the level of G-protein expression in a specific tissue may be related to the function of that tissue. Consequently, it is of interest to investigate whether drugs, such as morphine, can alter the normal levels of specific G-protein gene expression in specific brain tissues because it could indicate that the function of those tissue has been altered. In this study, we have measured mRNA levels as a method for studying gene expression. Changes in mRNA level often, but not exclusively, reflect changes in gene expression. The latter may result in changes in protein levels, which can cause functional changes. Measurements of mRNA levels are thus a convenient way to investigate whether molecular changes are occurring, but further investigations are required to determine the mechanisms of the changes of mRNA levels and whether they result in changes of protein levels and changes in physiological function.

In this study, we have detected changes in G-protein mRNA levels in the prefrontal cortex following both acute and chronic morphine administration. We did not detect similar significant changes in the nucleus accumbens, striatum or locus coeruleus. Although a number of reports have detected changes of some subunits in these tissues, these reports are not fully concordant with each other (Nestler et al., 1989; Terwilliger et al., 1991; Parolaro et al., 1993). One reason for differences among these studies could be that the regimes of morphine injection adopted may lead to different results with respect to G-protein mRNA levels. Although we have not directly measured the tolerance level of the morphine-treated rats, the similarity of our injection schedule to that of other studies leads to predict that the rats would have developed tolerance to morphine. In a preliminary study in which the chronic treatment was carried out for only 7 days compared with 14 days, we detected no changes in G-protein mRNA level even in the prefrontal cortex. Rats generally develop a high degree of tolerance after a few days of morphine injection; therefore, the observed changes in G-protein mRNA levels cannot be interpreted as a direct measure of tolerance induction, but probably represent an event which is part of the whole complex phenomena of drug addiction. An alternative explanation for the apparent lack of changes in G-protein mRNA levels in tissues other than the prefrontal cortex may be that our technique is less sensitive than the one used by other investigators and/or that a larger number of animals may have been required to obtain statistically significant changes in other brain regions. However, opioid receptors are associated with $G\alpha_{i1/2}$ in many brain regions and further studies will be required to explain why changes in $G\alpha_{i1/2}$ were observed only in the prefrontal cortex. It is also not possible to exclude the possibility that changes in G-protein mRNA were masked by a broad spectrum change of mRNAs that included both actin and GAPDH mRNAs, which were used as control house-keeping genes.

The observed changes in G-protein mRNA levels could have an important physiological role. The prefrontal cortex receives innervations from the ventral tegmental area, which has been implicated in reward behaviour (Koob, 1992). Additionally, G-protein metabolism in the prefrontal cortex could be directly affected by the activation of the opioid receptors in this tissue. Irrespective of the mechanisms by which they occur, changes of G-protein gene expression could have an effect on some of the normal functions of the prefrontal cortex. The large observed increase in $G\alpha_{i1}$ and $G\alpha_{i2}$ mRNA levels induced by chronic morphine injection could lead to an increase in the number of G-protein coupled receptors associated with these two G-protein subunits at resting state and a parallel decrease of G-protein coupled receptors associated to other G-protein subunits such as $G\alpha_o$. Upon receptor stimulation, in animals chronically treated with morphine, a greater number of $G\alpha_{i1}$ and $G\alpha_{i2}$ subunits and a smaller number of other subtypes of G-protein subunits would be released and a different population of specific effectors would be activated. Thus, the same external or internal stimulus would result in a different response in morphine-treated animals as compared to nontreated animals. Such differences could be related to behaviours associated with drug addiction.

Changes in gene expression of specific G-proteins not only could be associated with behavioural changes while subjects are under the effect of the morphine; however, if the changes were sustained, they could also play a role in other drug-related behaviours such as withdrawal and relapse into drug use. However, measurements carried out 48 h after termination of chronic morphine injection revealed that G-protein mRNA levels had returned to control levels. This would indicate that changes in G-protein gene expression are not associated with the molecular and behavioural long-term changes associated with chronic administration of morphine, which persist after termination of drug administration. Our data also suggests that chronic morphine-induced upregulation of the cAMP pathway (Nestler and Aghajanian, 1997) is not caused by changes in G-protein expression.

In summary, this work has presented an alternative methodology for the study of changes in G-protein gene expression in experimental drug addiction studies. In the prefrontal cortex, it has confirmed some of the previously reported observations about changes in subtypes of Gα subunits and determined, to our knowledge for the first time, morphine-induced changes in Gβ mRNA levels in the prefrontal cortex. Additionally, it has also shown that these changes in G-protein are short lasting. Further work is necessary to determine whether these changes in mRNA levels translate into an altered G-protein levels and altered receptor/G-protein association, which would have important physiological consequences and would offer an insight into at least some of the molecular mechanisms associated with drug addiction.

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