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Does the presence of morphine counteract adaptive changes in expression of G-protein α subunits mRNA induced by chronic morphine treatment?

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Abstract:
Opiate dependence develops due to changes in intracellular signaling caused by long-term exposure to morphine. Here we investigated changes in the mRNA expression of the main classes of G-protein alpha (Gα) subunits in various brain regions in morphine-dependent rats. Rats received increasing doses of morphine, 10–50 mg/kg, b.i.d., for 14 days. G-protein alpha-subunit mRNA expression was determined shortly following the conclusion of chronic morphine administration (2 h after the final dose) and during withdrawal (48 h after the final dose). Significant changes in mRNA expression for Gα subunits were observed in several brain areas during withdrawal, while the changes were much less evident or absent 2 h after the final drug injection. Changes in mRNA expression were particularly evident in the nucleus accumbens (increases in Gα(12), Gα(q), Gα(11), and Gα(o) during withdrawal, increase in Gα(i) and decrease in Gα(s) just following treatment). The direction of the changes, which were not all significant, for Gα(12), Gα(q), and Gα(11) was generally consistent in the amygdala and prefrontal cortex; changes in G proteins coupled to the adenylyl cyclase cascade were less consistent. These results suggest that morphine dependence leads to alterations in intracellular signaling, which are reflected in changes in the expression of genes encoding various G proteins. The results may explain why signs of opiate dependence are not expressed during chronic administration of morphine, but only after cessation of the treatment.

Key words:
chronic morphine, withdrawal, rat brain areas, G-protein alpha subunits, mRNA

Abbreviations: AC – adenylyl cyclase, AMY – amygdala, cAMP – 3'-5'-cyclic adenosine monophosphate, GPCR – the 7-transmembrane G-protein coupled receptors, NACC – nucleus accumbens, PFC – prefrontal cortex, PLC – phospholipase C β,

Introduction

A cell receives chemical signals by means of specialized membrane domains of which the most prevalent are the 7-transmembrane G-protein coupled receptors (GPCR). G proteins play a crucial role in linking receptor binding to changes in specific intracellular signaling pathways, whose signaling ultimately affects the regulation of genomic changes. Such changes are particularly important for phenomena of tolerance and dependence, in which opioid receptors are also engaged. All of the several known classes of opioid receptors belong to the GPCR superfamily (for review see [42]).

G-proteins are versatile signaling devices. They consist of three subunits: α, β, and γ. The α subunit is

the most important component for activation of specific signaling cascades. The Gα subunits have been classified into four main families: G(s) (stimulates adenylyl cyclase, AC), G(i) and G(o) (inhibit AC), G(q) and G(11) (stimulate phospholipase C β, PLC), and G(12) (modulates the activity of so-called small G proteins, e.g., Rho protein) [18, 28]. All the principal opioid receptors, mu, delta, and kappa, are associated with Gα(i) and Gα(o) proteins, however, some actions of morphine may also be due to the effects of the βγ subunit complex, released in response to G protein activation [46].

Chronic administration of morphine causes profound, long-lasting behavioral and biochemical changes, which are the basis for the phenomena of opiate dependence and abstinence syndrome after opioid withdrawal. These prolonged changes, which are induced by alterations in receptor-activated intracellular signaling cascades affecting the nucleus, result in changes in the expression of signaling [2] and trafficking proteins [29] and neuronal and glial cytoskeleton components [23].

Collier and Francis [9] first suggested that morphine dependence resulted from changes in intracellular signaling. Numerous later papers indicated that morphine dependence was related to activation of AC and protein kinase A [21, 27]. Evidently, such activation requires the involvement of not only G proteins directly associated with opioid receptors, but also those functioning in other systems that are indirectly stimulated by the action of morphine. Because opioid receptors are present on several neuronal types, and may therefore interact with a variety of neurotransmitters – such as norepinephrine [44], serotonin [40], glutamate [48] and GABA [10] – some of the ultimate effects of morphine may result from indirect activation of other receptor systems. Thus, the morphine administration, leading to the induction of dependence, affects several neuronal pathways. In addition, due to the interference of morphine with other neurotransmitter systems, the expression of G proteins other than G(i) and G(o) may also be affected by the drug.

Although initiation of a signaling cascade seems to be of paramount importance to the induction of long-lasting changes by exogenous compounds, the literature on the effects of chronic morphine administration on G proteins is rather limited and pertains mostly to G(i) and G(o) proteins. Studies investigating the mRNA expression of genes encoding G proteins are particularly scarce. With this gap in the literature in mind, we undertook investigation of the effects of chronic morphine administration on mRNA expression of the α subunits of G(i) and G(o) proteins. Moreover, as morphine affects other neurotransmitter systems, we also investigated the mRNA expression of α subunits for the G protein subtypes G(s), G(q/11), and G(12). We were particularly interested in the changes developing at the beginning of morphine withdrawal in morphine-dependent rats, as this period seems to be critical for the development of craving, which can trigger relapse in morphine addicts. To monitor the state of morphine dependence and abstinence we used behavioral techniques. We recorded the locomotor activity, which is elevated in morphine-dependent rats [4], and the body weight, whose gain is inhibited by chronic morphine administration and whose loss is precipitated by diarrhea, which is one of the signs of withdrawal syndrome [38].

Materials and Methods

Animals

Male Wistar rats (initial body weight between 190–210 g) were housed 4–6 to a cage at an ambient temperature of 21–23°C under a 12-h light/dark cycle (lights on at 06:00 h). Animals were randomly divided into experimental groups and were allowed 12 days of habituation before any treatment began. Food and water were available *ad libitum* except during locomotor testing. Half of the animals were used for behavioral tests and the second half for investigation of G protein mRNA expression. The behavioral and molecular experiments were run in parallel during the light period.

All experiments were carried out according to the NIH Guidelines for Care and Use of Laboratory Animals and were approved by the 2nd Local Ethics Commission at the Institute of Pharmacology, Polish Academy of Sciences in Kraków.

Morphine treatment protocol

A standard protocol to induce opiate dependence in rats was used [32, 39]. Briefly, morphine HCl (Polfa, Poland) dissolved in water was administered intrape-

ritoneally (*ip*) in a volume of 2 ml/kg. Rats were administered morphine twice daily, at 9:00 am and 6:00 pm, for 14 days. Treatment was initiated at a dose of 10 mg/kg (free base) twice daily. Beginning on treatment day 5, the dose was increased by 10 mg/kg every two days and then sustained at the maximal dose of 50 mg/kg twice daily on days 11–13. On day 14, the rats received only a morning injection. The control rats received saline injection.

Experiment 1: Effect of morphine treatment and withdrawal on locomotor activity

Locomotor activity was measured in clear plexiglass chambers (43 × 43 × 25 cm) housed inside Opto-Varimex® activity monitors. Rats were placed singly into a chamber and their locomotor activity was recorded by the control software AutoTrack System volume 3.30 (Columbus Instruments, Columbus, OH, USA). Crossovers, which registered upon breaking of 3 consecutive photobeams, were used as a measure of horizontal locomotor activity, defined as the distance traveled in cm.

Each test session began with a 1-h habituation to the testing chamber after which rats received an *ip* injection of morphine or saline and were immediately returned to the test chamber. Locomotor activity was recorded between the 60th and 120th minute after the injection. Following the test period, animals were returned to their home cages. Locomotor activity was again recorded between the 48th and 49th hour after the injection. Rats were weighed daily between 8:00 and 9:00 am. The sharp decline in the body weight next day after cessation of morphine administration, caused by excessive diarrhea, was regarded as a sign of withdrawal.

Experiment 2: Effect of morphine treatment and withdrawal on G-protein alpha subunit mRNA

Brain dissection

Rats in the short and long withdrawal groups were decapitated at 2 and 48 h, respectively, after the last injection. Brains were rapidly removed and placed on an ice-cold porcelain plate, and the prefrontal cortex (PFC), amygdala (AMY), and nucleus accumbens (NACC) were removed in anatomical limits using a visually guided procedure. The excised brain was placed upside down. The olfactory bulbs were removed, and the rest of the brain was coronally cut into two pieces 2 mm caudal to the middle cerebral artery. The NACC was removed in its natural limits from the frontal portion of the brain. All the subcortical structures were then removed and the remaining PFC taken. From the caudal portion a block of tissue containing the amygdalar nuclei was dissected as described by Engel and Sharpless [11]. The tissue approximately 0.5 mm below the rhinal fissure, inclusive of the majority of the amygdalar nuclei located between the pyriform cortex on one side and the optic tract and ventral portion of the lateral ventricles, substantia innominata, and ventral border of the caudate putamen on the other side, was dissected out. Tissue samples were frozen in liquid nitrogen and kept at –70°C until use.

RNA isolation

Total RNA was isolated and purified by the single-step method [7], with later modification [8]. Brain samples were placed in TriZol reagent (Invitrogen, Carlsbad, CA, USA) in a volume of 1 ml/100 mg of tissue and homogenized in a Teflon-glass homogenizer (Glass-Col, Terra Haute, IN, USA). RNA was precipitated using isopropanol (Sigma, St. Louis, MO, USA), added alone or together with glycogen (Invitrogen) for small structures (e.g. the NACC), as recommended by the TriZol manufacturer's protocol. RNA pellets were dissolved in diethyl pyrocarbonate-treated water (DEPC; Amresco, Solon, OH, USA), and the concentration of total RNA in the samples was assessed by spectrophotometry (Pharmacia Ultrospec 2000UV/Vis) at 260 nm for RNA, and 280 and 320 nm for DNA and protein contamination, respectively. RNA integrity was verified by gel electrophoresis (1% Agarose I; Amresco, Solon, OH, USA).

Synthesis of complementary DNA from mRNA (reverse transcription)

First DNA strand synthesis was achieved by reverse transcription (RT). RNA and 2 U of ribonuclease inhibitor (Fermentas, Vilnius, Lithuania) were incubated at 65°C for 5 min in 0.2-ml nuclease-free tubes (Eppendorf, Hamburg, Germany). The RT reaction was performed in a final volume of 20 µl containing 1X AMV reverse transcriptase buffer (50 mM Tris, pH 8.3; 10 mM $MgCl_2$; 50 mM KCl; 0.5 mM sper-

midine; 10mM 1, 4-dithiothreitol, Amresco), 1 mM deoxynucleotide-3-phosphate mixture (dNTP; Fermentas, Vilnius, Lithuania), 1 μM of each specific 3' primer (for each G protein and the housekeeping genes β-actin and hypoxanthine-guanine phosphoribosyltransferase, HPRT). Tubes were incubated at 25°C for 5 min, after which 10U of avian myeloblastosis virus reverse transcriptase (AMV, Amresco) was added. cDNA was synthesized at 42°C for 60 min, followed by a cycle of 70°C for 10 min. cDNAs for all genes, except Gα(i), were synthesized in a one-tube reaction. The RT products were stored at –20°C until further use.

The initial amounts of RNA (indicated in Figs.' legends) were in a linear relationship with final RT-PCR products for the given Gα subunit following the number of amplification cycles (as described below).

Amplification of cDNA (polymerase chain reaction)

A 2-μl volume of the RT product (RT solution containing cDNA) was amplified by polymerase chain reaction (PCR) with sense and antisense primers. cDNAs for Gα(q), Gα(11), and Gα(12) were simultaneously amplified in the multiplex PCR. No competition between primer pairs and individual PCR products was observed. Separate reactions were performed for other genes. The reaction mixture was composed of 1X PCR buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$, 0.1% Triton X–100; Finnzymes OY, Finland), 0.2 mM dNTP (Fermentas), 0.4 μM 5'-primer, 0.4 μM 3'-primer, and 0.5 U of DyNAZyme II DNA polymerase (Finnzymes). All incubations were conducted in a T3 Thermocycler (Biometra 2000, Germany). The incubation parameters and primer sequences are described below.

Gα(q), Gα(11) and *Gα*(12). An initial incubation at 94°C for 5 min was followed by 26 cycles (for PFC and AMY samples) or 22 cycles (for NACC) of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. The primers' nucleotide sequences were: 5' primer for Gα(q) 5'-GGAGGAGAGCAAAGCACTCTTTA (GenBank accession NM_031036); 5' primer for Gα(11) 5'-GGACCTTCTGGAAGACAAGATCC (GenBank accession AF239674); 5' primer for Gα(12) 5' TTCCAGTGCTTCGACGGGA (GenBank accession NM_031034), and 3' primer for all three Gα proteins 5' GAAGCGGATGTTCTCGGTGTC. Lengths of the multiplex RT-PCR reaction products for Gα(q), Gα(11), Gα(12) were 274, 190, and 369 bp, respectively.

Gα(o). An initial incubation at 94°C for 5 min was followed by 28 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. The primers' nucleotide sequences were: 5' primer 5' AACAAGTTTTTCATCGATAC and 3'-primer 5' TTACAAAGGCCAAAGGTCAT (GenBank accession M17526). These primers amplify cDNA fragments for two transcripts of different lengths, Gα(oA) (390 bp) and Gα(oB) (340 bp).

Gα(i). An initial incubation at 94°C for 5 min was followed by 28 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 15 min. Homologous cDNA fragments of α (i1), α (i2), and α (i3) subunits were simultaneously amplified in a multiplex RT-PCR reaction using a set of degenerate primers: 5'-primer 5'-CHATYGTSAARCAGATGA and 3'-primer 5'-AAARCAGTGRATCCACTT [34]. The primers were complementary to the two regions of Gα (i) showing extremely high DNA homology. Equimolar amounts of H (C, T, or A), S (G or C), R (A or C), or Y (C or T) nucleotides were incorporated during primer synthesis at the oligonucleotide position corresponding to mismatches in the sequences of Gα(i1), Gα(i2), and Gα(i3) genes (GenBank accession numbers: M17527, M17528, and M20713, respectively). These primers amplified cDNA fragments of equal length for Gα(i1) and Gα(i3) transcripts (503 base pairs), and a 506 bp segment of Gα(i2) mRNA. In order to distinguish the products of separate Gα(i) genes, the reaction products were digested with PstI restriction endonuclease. The digestion reaction mixture was composed of 1X buffer 0^+ (Fermentas), 3 U PstI restriction endonuclease (Fermentas), and 20 μl PCR product. Samples were then incubated at 37°C for 18 h. Restriction fragments of Gα(i) cDNA had different lengths that were separated by polyacrylamide gel electrophoresis. Products of Gα(i1) (length 476 and 27 bp) and Gα(i2) (373 and 133 bp) cDNA were then quantified.

Gα(s). An initial incubation at 94°C for 5 min was followed by 3 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and by 25 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 15 min. Primer sequences for the Gα(s) gene (GenBank accession: M12673) were: 5'-primer 5'-AGCAGCTGCAGAAGGACAAG and 3'-primer 3'-AGTCAGGCACGTTCATCACAC. Products of Gα(s) had lengths of 268 and 335 bp for short

α(s-s) and long α(s-l) splicing variants of the Gα(s) gene, respectively.

Control genes. Amplification of HPRT and β-actin was conducted as a qualitative internal control. The HPRT primer sequences (GenBank accession: AF001282) were sense primer 5'-GTCAACGGG-GGACATAAAAGT, antisense primer 5'-CAAGGG-CATATCCAACAACA; the PCR product was 254 bp in length. The primer sequences for the β-actin gene (GenBank accession: V01217) were sense primer 5'-CGTTGACATCCGTAAAGACC, antisense primer 5'-ACTCCTGCTTGCTGATCCAC; the PCR product was 226 bp in length. An initial incubation at 94°C for 5 min was followed by 18 or 20 cycles (for -actin and HPRT, respectively) at 94°C for 1 min, 60°C or 64°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min.

Primer sequences were based upon the sequences retrieved from the NCBI GenBank database and were designed using OLIGO Primer Analysis Software (version 5.0, NBI). To rule out genomic DNA contamination in the PCR amplification, the primers were chosen in different exons of each gene. All primers were custom synthesized and purchased from TIB Molbiol (Poland).

Agarose gel electrophoresis

For assessment of Gα(12), Gα(q), Gα(i1), and Gα(oA), PCR products were electrophoresed in a non-denaturating 1.3% agarose gel (super-fine resolution, SFR agarose; Amresco) containing 5×10^{-4}% ethidium bromide. A 10 μl volume of PCR product composed of PCR reaction solution (84% v/v) combined with (16% v/v) loading buffer (0.5X Loading Dye in 50% sucrose, Fermentas) was pipetted onto each well of the gel. In each gel, one lane was loaded with Gene Ruler 50 bp DNA (Fermentas) or PhiX174 DNA *BsuRI* (*HaeIII*) ladder. Electrophoresis was performed in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA; Amresco, Solon, OH, USA), at 75 V for 2.5 h. PCR products were visualized using a CCD camera (Fuji Las 1000), and densitometric quantification of the fluorescence bands was carried out using Image Gauge 4.0 software (Fuji).

Values were recalculated as a ratio of the Gα subunit band intensity divided by the intensity of the reference gene amplified from the same RNA sample.

Polyacrylamide gel electrophoresis

For assessment of Gα(s) and Gα(i), the PCR and digestion products were separated by polyacrylamide gel electrophoresis. Band intensity was assessed by laser fluorometry using YOYO-3 (Molecular Probes, Eugene, OR, USA), an intercalating fluorescent dye, and an automated electrophoresis unit (Alf-Express II, Pharmacia, Uppsala, Sweden). Each gel was composed of 0.5X TBE buffer, 5% Acryl/Bis 29 : 1 Solution, 2.6 mM ammonium persulfate, and 0.1% TEMED (Amresco).

Samples were pipetted into each well of the gel in a total loading volume of 4 μl, composed of 3 μl of digestion- or PCR-products combined with 1 μl of loading buffer (50% sucrose; 1 μM YOYO-3; and 0.4 or 2 fmol of loading marker, the 100-bp DNA fragment, ALFexpress Sizer 100) (Amersham Pharmacia Biotech, NJ, USA). In each gel, one lane was loaded with Gene Ruler 50-bp DNA Ladder (Fermentas). The gels were run at 100 mA, 500 V, and 25°C for 100 min. Areas under the peak of cDNA and loading marker were determined in automated fashion using ALFwin Fragment Analyser software (Pharmacia). The absolute fluorescence signal was recalculated as the ratio of cDNA to loading marker, and either HPRT or β-actin was used as a reference gene.

Statistical analysis

All values are averages of five or six rats ± the standard error of the mean (SEM). Statistical analysis was performed using Statistica 5.0 software (StatSoft, Tulsa, OK, USA) using one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test or Student's *t*-test (body weight analysis); $p < 0.05$ was considered statistically significant.

Results

Effect of morphine treatment and withdrawal on locomotor activity

The body weight gain in rats receiving morphine was inhibited (Fig. 1). Cessation of treatment caused a sharp drop in body weight (Fig. 1), accompanied by transient but excessive diarrhea.

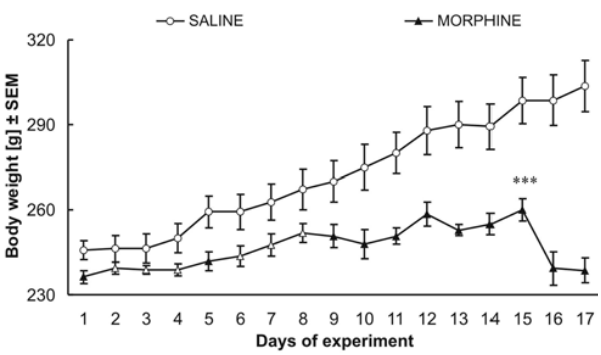

Fig. 1. Body-weight gain during chronic morphine treatment and withdrawal. The initial mean body weight ± SEM was 247.1 ± 3.4 g (saline controls) and 236.4 ± 2.6 g (morphine group). Each point represents the mean ± SEM (n = 7). Circles, saline controls; triangles, morphine-treated rats. Closed symbols show a statistically significant difference between the saline- and morphine-injected group (Student's *t*-test; $p < 0.05$). Asterisks denote a significant difference between day 15 and subsequent days at $p < 0.001$ (Fischer's LSD test)

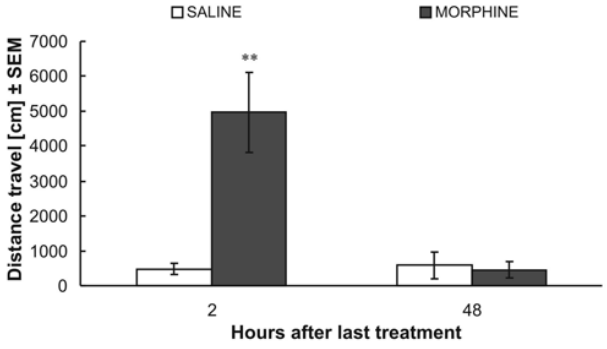

Fig. 2. Locomotor activity of rats following chronic morphine treatment and 48 h after morphine withdrawal. Locomotor activity was recorded for 1 h when animals were under the influence of morphine (starting 1 h after morphine injection) and during the period of withdrawal (48 h after the final dose of morphine). Each bar represents the mean ± SEM (n = 7). White bars, saline groups; shaded bars, morphine-treated rats. Asterisk denotes a significant difference between saline- and morphine-treated rats [$F(1,12) = 15.59$, $p < 0.01$, one-way ANOVA

Successive doses of morphine caused a progressive increase in locomotor activity (data not shown). The final dose (50 mg/kg) induced an approximately 10-fold increase in locomotor activity, peaking between 60 and 120 min after the injection and lasting for over 2.5 h. Motor activity 48 h after the last injection was similar in control and morphine-dependent rats (Fig. 2).

Effect of morphine treatment and withdrawal on expression of G-protein αsubunit mRNA

*G*α(q), *G*α(11), and *G*α(12)

After a short withdrawal period no significant changes in the expression of mRNA encoding Gα(q) and Gα(11) proteins were observed (Fig. 3 A–C). Elevation of Gα(12) in the NACC (approximately 40%) also did not reach statistical significance at this time (Fig. 3 C).

In contrast, after long withdrawal significant increases in the expression of Gα were observed in the PFC [$F(2,15) = 4.89$, $p < 0.023$ for G(12); $F(2,15) = 8.23$, $p < 0.0038$ for G(11)] (Fig. 3 A) and in the NACC [$F(2,13) = 3.919$, $p < 0.046$ for G(12); $F(2,14) = 5.71$, $p < 0.01$ for G(q); $F(2,13) = 4.159$, $p < 0.04$ for G(11)] (Fig. 3 C). In the AMY no changes reached statistical significance (Fig. 3 B).

*G*α(o)

The primers used in this experiment were designed to recognize two isoforms of the Gα(o) subunit, α(oA) and α(oB). These isoforms arise from alternative splicing of RNAs from a single α(o) gene [43]. We were able to detect only the longer variant, Gα(oA) (390 bp), which was shown to be predominant in the rat brain [24].

The only significant change in Gα(o) mRNA expression was a marked increase in Gα(oA) expression in the NACC during the withdrawal period [$F(2,13) = 3.919$, $p < 0.05$] (Fig. 4 C). No changes were observed in the PFC and AMY at either time (Fig. 4 A and B).

*G*α(i-1) and *G*α(i-2)

Expression of both Gα(i1) and Gα(i2) in the AMY was depressed (by approximately 50%) after chronic morphine treatment both 2 h and 48 h after the last morphine dose, though only the changes for Gα(i-2) at 48 h reached statistical significance [$F(2,13) = 2.116$, $p = 0.16$, LSD $p < 0.087$] (Fig. 5 A and B). In contrast, expression of Gα(i1) and Gα(i2) was significantly elevated in the NACC 2 h after the last dose of morphine [for Gα(i1) $F(2,13) = 2.51$, $p < 0.118$, LSD $p = 0.055$; for Gα(i2) $F(2,13) = 2.805$, $p < 0.097$; LSD $p < 0.05$], but normalized by 48 h after morphine (Fig.

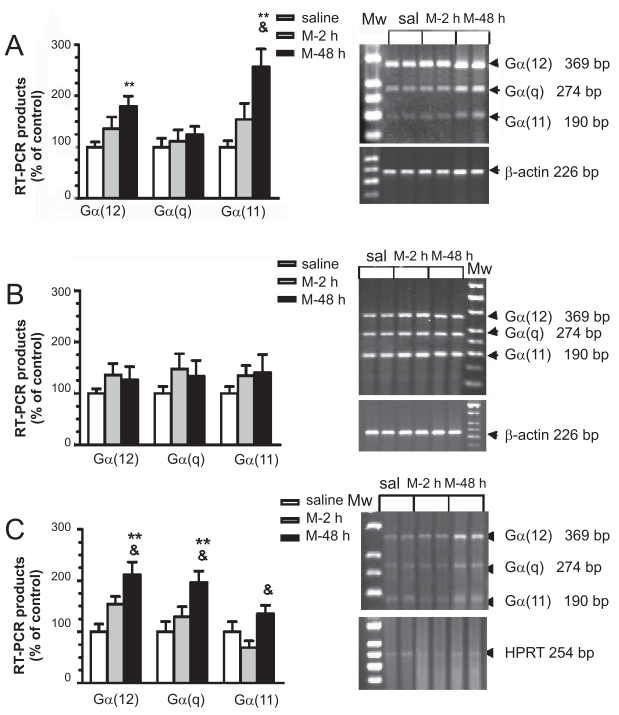

Fig. 3. Changes in mRNA expression of Gα(12), Gα(q), and Gα(11) in prefrontal cortex (**A**), amygdala (**B**) and nucleus accumbens (**C**) of rats after withdrawal from chronic morphine. Animals were decapitated 2 h (M-2h) or 48 h (M-48h) after the last injection of morphine or saline. Data are expressed as a percent of control value (saline-treated rats), and bars represent the means ± SEM (n = 5–6). Inserts show the representative gel electrophoresis for simultaneous detection of three Gα subunits. A 1.5 μg (prefrontal cortex and amygdala) or 0.6 μg (nucleus accumbens) of total RNA was transcribed to cDNA. Mw, marker of molecular weights; ** $p < 0.01$ and $^{\&}$ $p < 0.05$ compared to the saline or M-2h group, respectively (Fisher's LSD test)

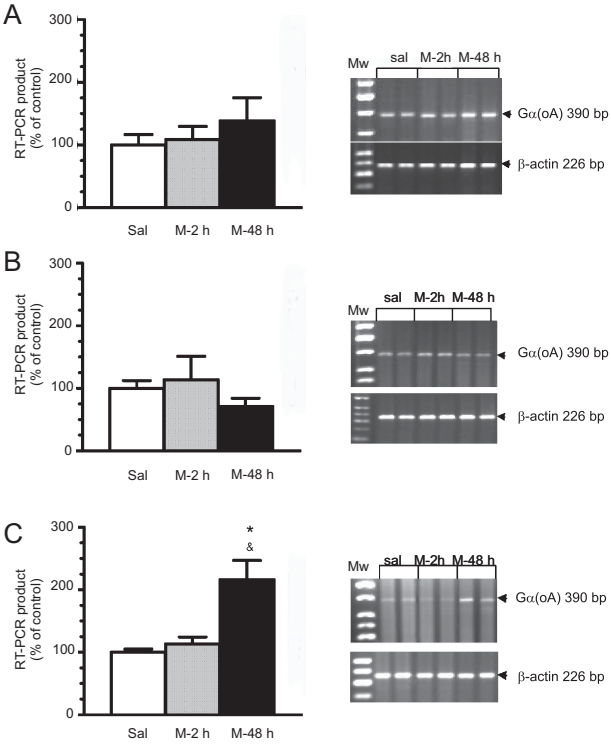

Fig. 4. Effects of chronic treatment with morphine and morphine withdrawal on expression of Gα(oA) mRNA in prefrontal cortex (**A**), amygdala (**B**) and nucleus accumbens (**C**) of rats. M-2h and M-48h correspond to animals decapitated 2 h and 48 h after the last injection of morphine, respectively; sal, saline control group. Data are expressed as a percent of saline value, and bars represent the means ± SEM (n = 4–6). Inserts show the representative gel electrophoresis for Gα(oA) and β-actin in each brain structure. A 1.5 μg (prefrontal cortex and amygdala) and 1.0 μg (nucleus accumbens) of total RNA was reversely transcribed to cDNA. Mw, marker of molecular weights; * and $^{\&}$ designate $p < 0.05$ significant difference from the saline or M-2h group, respectively (Fisher's LSD test)

5 C and D). No changes were observed in the PFC (data not shown)

Gα(s)

Primers used in our experiment recognized two splicing variants of *Gα*(s): the short form, Gα(s-s), and the long version, Gα(s-l). The latter variant was reported to be most abundant in rat brain [13]. We found no changes in the expression of the short variant Gα(s-s) following chronic morphine treatment or withdrawal. Changes in the long variant, Gα(s-l), were transient (no differences after 48 h) and nonuniform. Thus, 2 h after the last dose of morphine there was a borderline increase [$F(2,14) = 2.00$, $p = 0.17$; LSD test $p < 0.065$] in the PFC (Fig. 6 A), a significant decrease [$F(2,13) = 3.93$, $p = 0.046$; LSD $p < 0.01$] in the NACC (Fig. 6 C), and no change in the AMY (Fig. 6 B).

Discussion

The neurochemical effects even of a morphine single dose are very complex. This complexity is reflected in the multiplicity of morphine's behavioral effects, which vary from sedation to running fit, depending on the animal strain [5] and the time elapsed since administration of the drug [22]. We confirmed that the motor effects of morphine increase during chronic treatment (see [1]).

The effects of chronic morphine administration are much more complicated. In rats repeated morphine administration leads to rapid development of dependence and complex behavioral changes that vary according to the length of the withdrawal period. In hu-

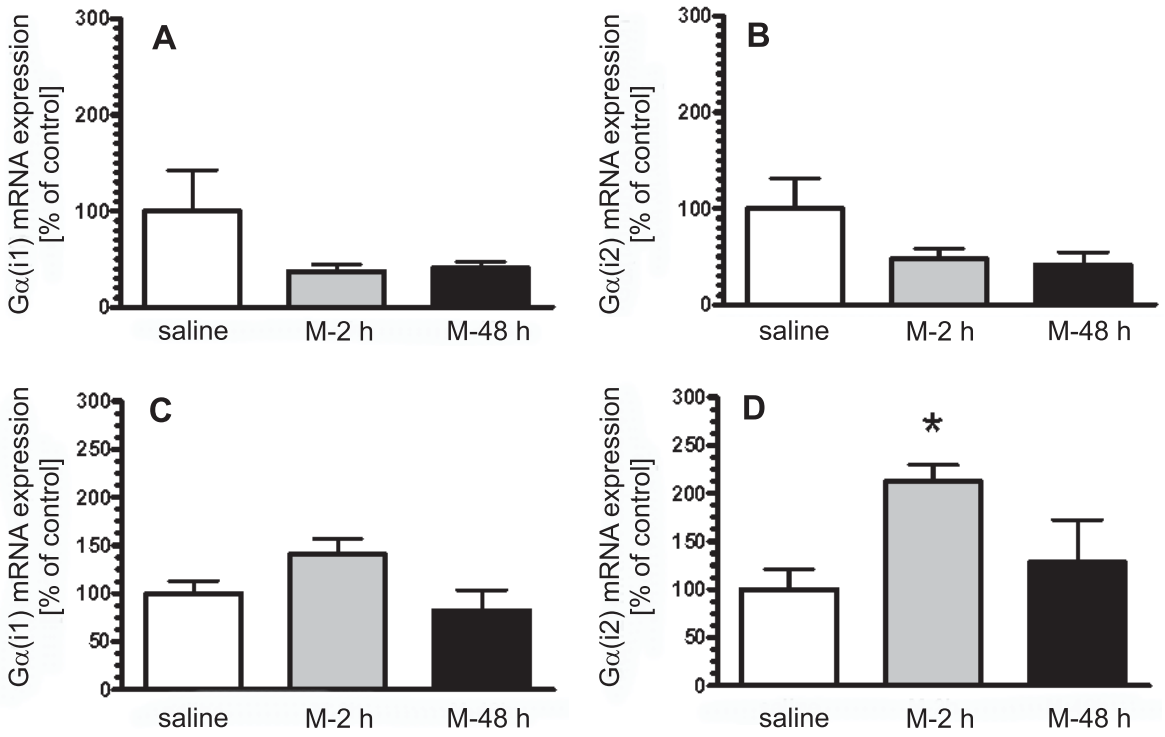

Fig. 5. Effects of chronic treatment with morphine and morphine withdrawal on mRNA expression of Gα(i-1) and Gα(i-2) subunits in the amygdala (**A** and **B**), and the nucleus accumbens (**C** and **D**) of rats. M-2h and M-48h correspond to animals decapitated 2 h and 48 h after the last injection of morphine, respectively. A 1.5 μg (amygdala) and 2.0 μg (nucleus accumbens) of total RNA was reversely transcribed to cDNA. Results are expressed as a percent of control value (saline-treated rats), and bars represent the means ± SEM (n = 4–6). * $p < 0.05$ *vs.* saline group (Fisher's LSD test)

mans, morphine abstinence results in strong psychological effects (craving) that may be alleviated by a single dose of morphine. Similarly, abstinent animals display search behavior when morphine availability is expected, and administration of morphine relieves the behavioral symptoms of abstinence and appears to normalize behavior. Thus, chronic morphine administration does not elicit strong behavioral changes when the drug is administered regularly, although some adverse health effects develop. The inhibition of body weight gain and increased motor response observed in this study indicate that chronic morphine treatment resulted in profound alteration of the animals' physiological state. The cessation of morphine administration results in severe physiological and behavioral disturbances manifesting as diarrhea and increased irritability.

We theorized that the adaptive changes induced by chronic administration of morphine developed progressively during treatment, but were masked by morphine administration. Assuming that changes in G protein signaling may serve as a marker for these adaptive changes, we assessed G protein mRNA expression at two time intervals following chronic morphine administration: 2 h after the final dose of morphine (i.e. at the time of high morphine concentration in the brain and demonstrable behavioral response) and 46-h later (when the morphine-induced motor stimulation was absent and withdrawal symptoms were strongly expressed). Although changes in mRNA levels not necessarily parallel the changes in protein levels, several authors assume that generally mRNA changes reflect functional changes. We therefore believe that the changes in mRNA expression for various types of G proteins may reflect altered functioning of signaling cascades. The major finding was that mRNA encoding several G-protein α subunits in various brain areas was more highly expressed at 48 h than at 2 h after the last dose of morphine.

In particular, we have described for the first time that morphine withdrawal induces an increase in Gα(12), Gα(q), and Gα(11) mRNA expression, and that these changes are much less evident – or absent – shortly after the last morphine injection, when mor-

phine is present in the brain and can antagonize the adaptive changes induced by chronic morphine injection that lead to withdrawal syndrome. The direction of these changes in mRNA expression was generally consistent in all brain structures examined. The late appearance and topographic specificity of the morphine-induced changes in mRNA expression of Gα(q/11) and Gα(12) confirm and extend those described for protein expression in morphine-treated mice based on Western blot experiments [26, 47].

It should be underlined that there is no evidence indicating that Gα(12), Gα(q) and Gα(11) proteins are coupled directly to opioid receptors. Nevertheless, using other G protein subunits, opioid receptors may regulate PLC, an effector enzyme downstream of Gα(q/11) [42]. Gα(12) and Gα(q/11) are coupled to distinct signaling pathways. Gα(12) (together with Gα(13)) regulates the activity of small Rho protein through RhoGEF [36], while Gα(q/11) stimulates PLC β [28]. Nevertheless, the Gα(12/13)- and Gα(q/11)-mediated pathways may cooperate, and in some situations their interaction is necessary for the induction of cellular plasticity. Thus, Gα(12/13)-mediated pathways are essential for particular cellular responses induced by Gα(q)-coupled receptors (see [18]). The role of such an interaction in the central nervous system is not yet clear, but might possibly affect neuronal plasticity, as Katoh et al. [16] demonstrated that activated Gα(q), Gα(12), and Gα(13) all induced Rho-dependent neurite retraction and cell rounding in differentiated PC12 cells.

As Gα(q/11) and Gα(12/13) proteins are not regulated by opioid receptors, the changes in their expression may have been induced by alteration of upstream receptors in response to chronic morphine administration/withdrawal. Receptors upstream of Gα(q/11) (e.g, noradrenergic and glutamatergic receptors [20, 31, 33]) and Gα(12) (e.g., neurokinin receptors [12]) have been shown to be part of morphine mechanism of action. In some cases, these receptors may help to regulate two distinct metabolic cascades (see [3, 30]). Therefore, the lack of response of one Gα protein (Gα(q) in the cortex or Gα(11) in the NACC) to chronic morphine administration or morphine withdrawal does not preclude the possibility that the appropriate cascade is activated by another Gα subtype. Studies by Jasmin et al. [14] suggest that such substitution of signal cascades involving Gα(q) and Gα(12) may account for the reduced opioid efficacy associated with deficits in noradrenergic transmission.

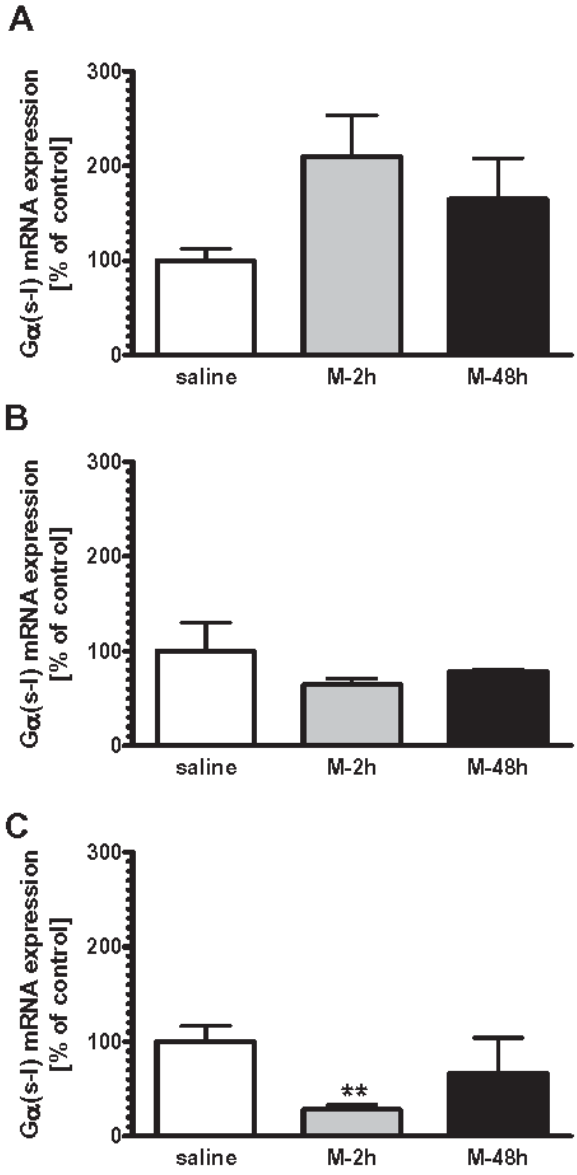

Fig. 6. Effects of chronic morphine treatment and morphine withdrawal on Gα(s-l) mRNA expression in the prefrontal cortex (**A**), amygdala (**B**), and nucleus accumbens (**C**) of rats. First strand DNA synthesis was achieved by reverse transcription of 1.5 μg of total RNA (prefrontal cortex and amygdala) or 1 μg (nucleus accumbens). Other details as for Fig. 5; n = 5–6; ** $p < 0.01$ *vs.* saline (Fisher's LSD test)

While the role for Gα(q) and Gα(12) in reward has not been considered in the literature, it may be proposed that G(i) and G(o) proteins in the NACC play a common role in the reinforcing and addictive properties of opiate and psychostimulant drugs, since inhibition of Gα(i) and Gα(o) produced long-lasting increases in heroin and cocaine self-administration in rats [35]. Also, the concentrations of Gα(i1) and/or

Gα(i2) were reduced by 32–49% in the NACC of methamphetamine and heroin users [25]. In contrast to Gα(q) and Gα(12) proteins, G(i) and G(o) proteins act downstream of opioid receptors, and although we found that they were also affected by chronic morphine administration, the changes were less consistent and confined to subcortical structures. The pattern of changes of genes encoding Gα(i) was different in the AMY and NACC. In the AMY, expression was diminished at both 2 and 48 h after the final morphine injection, suggesting that morphine administration was unable to reverse the change induced by the withdrawal state. In the NACC, however, morphine administration resulted in increased expression at 2 h, but not 46-h later. On the other hand, the expression of the Gα(o) protein significantly increased only in the NACC of 48 h morphine-withdrawn rats. This may be interpreted as an indication that the direct action of morphine counteracts the withdrawal-enhanced expression of Gα(o).

Although it is generally assumed that opioid receptors are linked predominantly with Gα(i) and Gα(o) proteins, recently Chakrabarti et al. [6] demonstrated the existence of a subset of mu receptors associated with Gα(s). In the present study, we found that expression of the Gα(s) protein mRNA, which, in contrast to Gα(i) and Gα(o) is coupled to stimulation of the cAMP cascade, was also affected by chronic morphine treatment and withdrawal. In the NACC the change in Gα(s) mRNA expression was opposite to that observed for the Gα(i) protein: a decrease 2 h after the final morphine injection and no difference from control at the 48-h time point. It was not changed in the AMY, but in the PFC expression was increased 2 h after the final morphine dose, and this change seemed to persist 48-h later (no significant difference from the 2-h value), although at this time point the level of expression was not significantly different from the control value.

It has been reported that morphine-induced changes in the G protein-coupled activation of the cAMP cascade are confined to specific brain areas [41] and that the development of changes in the expression of G protein mRNA or protein depends on the mode of morphine abstinence induction [15, 32, 45]. These observations are consistent with the present findings that changes in the mRNA expression of these proteins also show different distributions and, in some areas (particularly in the NACC), differ 2 and 48 h after the last dose of morphine.

The fact that the NACC is a critical area in the reward system did not escape our attention. Our analysis of changes in G protein mRNA expression in the NACC in the rewarded state (shortly after morphine injection in morphine-dependent rats) revealed a decrease in mRNA expression of Gα(s-l) and an increase in mRNA expression for Gα(i1) and Gα(i2). This suggests a decrease in the activity of the AC pathway. In contrast, during abstinence the expression pattern of Gα(s) and Gα(i) mRNA returned to normal. This suggests that the changes observed shortly after the final morphine injection are a direct consequence of the drug's action. The state of abstinence was marked by a profound increase in the expression of Gα(oA) mRNA. Our failure to find changes in Gα(oA) shortly after morphine (which is known to have strong reward value in this situation [37]) suggests that morphine injection completely reversed the effect of long-term morphine treatment in this respect. This may be analogous to the reversal of withdrawal syndrome in human addicts *via* opiate administration [19].

In contrast to the changes in the NACC, which were different in the presence and absence of morphine in the brain, the changes in Gα(i) mRNA in the AMY were similar at both time points. In light of the allostasis model of addiction proposed by Koob and Le Moal [17], these similarities may reflect the process of maintaining apparent reward function stability through changes in various neurotransmitter Gα(i)-coupled receptors that are involved in brain reward mechanisms. and thus may be of functional relevance.

In conclusion, our results suggest that chronic morphine administration leads to slow development of changes in intracellular signaling, which are reflected by changes in the expression of genes coding for various G proteins. The effects of these changes in gene expression may be, at least partially, blocked by the acute effects of continuously administered morphine. However, they may be easily observed during withdrawal 48-h after morphine administration, when they are confounded neither by the direct action of morphine (which at this time disappears from the brain) nor by potential later adaptive changes to the withdrawal state.

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