Cytoskeletal Genes Regulation by Chronic Morphine Treatment in Rat Striatum

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It has been previously suggested that morphine can regulate the expression and function of some proteins of the cytoskeleton. In the present study, we used real-time quantitative polymerase chain reaction to examine the effects of chronic morphine administration, in rat striatum, on 14 proteins involved in microtubule polymerization and stabilization, intracellular trafficking, and serving as markers of neuronal growth and degeneration. Chronic morphine treatment led to modulation of the mRNA level of seven of the 14 genes tested. Glial fibrillary acidic protein (*Gfap*) and activity-regulated cytoskeleton-associated protein (*Arc*) mRNA were upregulated, while growth associated protein (*Gap43*), clathrin heavy chain (*Cltc*), α -tubulin, Tau, and stathmin were downregulated. In order to determine if the regulation of an mRNA correlates with a modulation of the expression of the corresponding protein, immunoblot analyses were performed. With the exception of *Gap43*, the levels of *Cltc*, *Gfap*, Tau, stathmin, and α -tubulin proteins were found to be in good agreement with those from mRNA quantification. These results demonstrate that neuroadaptation to chronic morphine administration in rat striatum implies modifications of the expression pattern of several genes and proteins of the cytoskeleton and cytoskeleton-associated components.

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INTRODUCTION

There is increasing evidence that morphine alters gene expression in different areas of the brain, even following a single injection. Acute morphine administration induces modification of the expression of genes involved in processes as diverse as signal transduction coupled to μ opioid receptors (Fan *et al*, 2002, 2003; Kaewsuk *et al*, 2001; Przewlocka *et al*, 1994), transcription factors (Erdtmann-Vourliotis *et al*, 1998; Kelz *et al*, 1999), and calcium binding proteins (Tirumalai and Howells, 1994). Interestingly, an analysis of the gene expression profile following a single injection of morphine revealed that one of the major groups of altered genes in the rat medial striatum consisted of cytoskeleton-related proteins (Loguinov *et al*, 2001). Morphine has been shown to induce neuronal changes that persist for a long time following cessation of drug exposure (Nestler and Aghajanian, 1997). However, the molecular and cellular mechanisms underlying these long-lasting changes are still not fully understood.

Nevertheless, the most interesting results, which are expected to have functional implications in the long-lasting effects of morphine, are those obtained following chronic treatment. An increase in dynamin expression after chronic morphine treatment has been shown in mice striatum (Noble *et al*, 2000). This protein has a key role in receptor endocytosis and is able to interact with microtubules, actin cytoskeleton, and the mitogen-activated protein kinase signalling pathway (review in Sever, 2002). Among the remarkable changes observed following repetitive administration of morphine, a reduction in the size of dopaminergic neurons in the ventral tegmental area and a decrease in dendritic branching and spine density in the cerebral cortex and nucleus accumbens have been reported (Skair-Tavron *et al*, 1996; Robinson and Kolb, 1999). Consistent with these morphological changes, the expression and function of neurofilament proteins are altered by chronic morphine treatment in the ventral tegmental area in rats (Beitner-Johnson *et al*, 1992; Beitner-Johnson and Nestler, 1993). Alterations of neurofilaments have also been found in the prefrontal cortex of human opioid addicts (Garcia-Sevilla *et al*, 1997; Ferrer-Alcon *et al*, 2000). Moreover, a decrease in the volume of frontal cortex of male opioid-dependent patients has been reported by Pezawas *et al* (1998). Taken together, these results suggest that opiates may induce neuronal damages in humans, and that the modifications observed in rat models are pertinent to the investigation of this problem, thought to be the basis of morphine addiction.

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We therefore investigated the mRNA and protein expression levels of several cytoskeletal or cytoskeletal-associated proteins in rat striatum after repeated administration of increasing doses of morphine. This was achieved by combining real-time quantitative RT-PCR and immunoblot analyses. Markers of neuronal plasticity like growth-associated protein-43 (Gap43) and activity-regulated cytoskeleton-associated protein (Arc); of intracellular trafficking, like dynamin, clathrin heavy chain (Cltc) and filamin α (Flna) or of microtubule assembly and stabilization like, α -tubulin, stathmin, Stop, and Tau were chosen. Since active interactions between neuron and glia are important for synaptogenesis and synaptic plasticity (review in Bacci *et al*, 1999), we also studied glial fibrillary acidic protein (Gfap), a marker of astroglial cells. Modulations of mRNA and proteins levels were observed following chronic morphine administration supporting the hypothesis of a relationship between cytoskeleton modifications and long-lasting actions of morphine.

MATERIALS AND METHODS

Animals and Drug Treatment

Male Sprague–Dawley rats (IFFA CREDO, France) weighing 180–200 g at the beginning of the experiments were used. Experiments were performed according to the European Communities Council Directive (86/609/EEC). Animals were housed under controlled conditions (12 h alternating light/dark cycle at $21 \pm 1^\circ\text{C}$) and had free access to food and water. Saline and morphine (Francopia, France) were injected i.p. twice daily. Morphine was progressively increased from 10 to 40 mg/kg/dose. The first and second number inside parentheses represent the dose of morphine (mg/kg) injected at 0900 and 1900 hours, respectively, on consecutive days: first day (10,20), second day (20,40), third day through fifth day (40,40) as previously described (Noble *et al*, 2000). The animals were killed by decapitation on the

morning of the sixth day, 16 h after the last injection. This time point was chosen on the basis of previous studies using a similar protocol showing that the significant behavioral signs of spontaneous withdrawal appear 24 h after the last morphine injection (Ruiz *et al*, 1996). Different animals were used for real-time PCR analysis and immunoblotting. The striata were rapidly dissected on ice and frozen in isopentane at -50°C and stored at -80°C until use.

RNA Isolation and Real-Time PCR Analysis

Total RNA was extracted from individual striatum with RNABle reagent (Eurobio, France) following the manufacturer's protocol. The quality of total RNA was assessed by agarose gel and quantified by spectrophotometry. RNA was reverse transcribed in a final volume of 20 μl containing $1 \times$ RT-PCR buffer (3 mM MgCl_2 , 75 mM KCl, and 50 mM Tris-HCl (pH 8.3)), 500 μM each deoxynucleotide triphosphate, 20 U of RNasin RNase inhibitor (Promega, France), 10 mM DTT, 100 U of Superscript II RNase H⁻ reverse transcriptase (Invitrogen, France), 1.5 μM random hexamers (Amersham-Pharmacia, France), and 1 μg of total RNA. Samples were incubated at 20°C for 10 min and 42°C for 30 min, and reverse transcriptase was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min. PCR primers were chosen with the assistance of Oligo 6.42 software (MedProbe, Norway). The primer nucleotide sequences used in this study are listed in Table 1. Fluorescent PCR analysis was performed using a LightCyclerTM instrument (Roche Diagnostics, France). The cDNAs were diluted 40-fold and 5 μl were added to the PCR reaction mix to yield a total volume of 10 μl . The PCR reagents were obtained from the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, France). The reaction buffer contained 4 mM MgCl_2 and 0.5 μM of each primer. Amplification protocols consisted of 35–40 cycles of denaturation at 94°C (5 s), annealing at 68°C (5 s), and extension at 72°C (10 s). Fluorescent signals were obtained once in each cycle by sequential-fluorescence

Table 1 Primer Sequences Used for SYBR Green-Based Real-Time Quantitative Polymerase Chain Reaction

Gene	Forward primer	Reverse primer
Tbp	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
Flna	TGGCCAGCCTTCCATTACTG	GCCCCCTCTCCTGCATC
Gap43	GATGCGGCCCTTCAGAG	CCTTGGCTGGGCCATCTT
Gfap	CAGAACTCCAAGATGAAACCAA	TCTCCTCCTCCAGCGACTCAAC
Tau	ACCATGGCTTAAAAGCTGAAGAAG	CGGCCACTCGAGCTTGAGTC
Snca	TGGGCAAGGGTGAAGAAG	TAGGCTTCAGGCTCATAGTCT
Stathmin	CCGGGAGGCGCAAATG	TTGTTCTTCCGCACCTCTTCA
Cltc	ATCGCCAGCTGTGTGAGAA	CCACTCGGGGTTGAGAAGATG
Stop	GCGTGGCCGTGCATCACTAG	CGGGCAACTGCATCCGACT
α -tubulin	TTGAGCGCCCAACCTACACT	TCAGGGCCCCATCAAATCT
Fos	GGCAAAGTAGAGCAGCTATCTCCT	TCAGTCCCTCCTCCGATTG
Dynamin-I	CTTGCGGGGACCAGAACAC	GGCATGGGCGTGCTGAC
Arc	GCCGCCAAACCAATGTGA	GTCGCCGTGGGCACATAG
β -actin	CTGGCCGGGACCTGACAGA	GCGGCAGTGGCCATCTCAT
Synphilin-I	GGGCGAGCTGGAGCACTAT	GGGGCCAGCTGTTGACTG

monitoring of each sample tube at the end of extension. cDNAs from a naive rat brain were used to generate an external standard curve for each gene. We also quantified transcripts of the gene coding for the TATA-binding protein (Tbp), a component of the DNA-binding protein complex TFIIID, as the endogenous RNA control and normalized each sample on the basis of its Tbp content as previously described (Bieche *et al*, 2000).

Immunoblotting

Rat striata were homogenized in lysis buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 30 mM sodium pyrophosphate, 1 mM DTT, 5 μ M ZnCl₂, 1% triton, 50 mM NaF, 0.5 mM PMSF, 100 μ M sodium orthovanadate, 5 nM okadaic acid, and CompleteTM protease inhibitor complex (Roche Diagnostics, France). Homogenates were centrifuged at 4°C, 15 min at 15 000g, and the supernatants were used. The protein concentration of samples was determined using Bradford reagent (Sigma, France) and bovine serum albumine (BSA) as a standard. Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with primary antibody. The antibodies used were Gfap (1:2000), Gap43 (1:2000), and Tau (1:2000) from Chemicon (France); β -actin (1:10000) and α -tubulin (1:10000) from Sigma (France); Stathmin (1:20000) from Calbiochem (France); Clathrin (1:1000) and Arc (1:250) from BD Biosciences (France); Dynamin (1:6000) from Upstate (UK). Secondary antibodies were anti-mouse and anti-rabbit horseradish peroxidase-linked antibody (1:20000) from Amersham Bioscience (France) and anti-goat horseradish peroxidase-linked antibody (1:20000) from Jackson ImmunoResearch (France). Immunoreactive protein bands were detected by enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, France) on an X-ray film. Protein expression was quantified using Bio1D software (Vilber Lourmat, France).

Statistical Analyses

All series of data were analysed with GraphPad Prism[®] software. Results are displayed as mean $\pm$ SEM of 4–6 animals. Statistical analyses were performed using Student's test. The level of significance was chosen as $P < 0.05$.

RESULTS

Modulation of the mRNA Levels of Several Genes by Chronic Morphine Treatment in Rat Striatum

Using the real-time quantitative RT-PCR method, we investigated expression modulation in 14 cytoskeletal-related genes by chronic morphine administration (Figure 1). The expression levels of two immediate-early genes were assessed. The mRNA level of the immediate-early gene Fos was not affected by chronic morphine treatment. On the contrary, the immediate-early gene Arc was significantly upregulated by chronic morphine treatment (1.48-fold). Two genes involved in clathrin-coated pit formation were analyzed. *Cltc* mRNA level was significantly downregulated (1.4-fold) in morphine-treated rats, while that of dynamin showed a slight but not significant increase.

The expression levels of actin and of one of its associated proteins, Flna, were not affected by chronic morphine treatment. The expression levels of two proteins involved in the formation of cytoplasmic inclusions, Snca and Synphilin-1, were not affected in the striatum of rats chronically treated with morphine, as compared to control animals. The mRNA level of four genes involved in microtubules polymerization, stabilization, and depolymerisation were analyzed. A significant decrease was observed in three of them: stathmin, α -tubulin, and *Tau* (1.53-, 1.56- and 1.42-fold, respectively) following chronic morphine treatment, while the expression level of the microtubule stabilizer Stop was not altered under these conditions. Chronic morphine treatment led to a significant increase of the mRNA level of the astroglial specific marker Gfap in the striatum (1.41-fold) as compared to control animals. The mRNA level of the neuronal growth cones marker Gap43 was significantly decreased in morphine-treated rats (1.42-fold). In order to determine if regulation of the mRNA level of these genes by chronic morphine could lead to regulation of the expression of the corresponding proteins and thus to have a functional impact, immunoblot analyses were performed.

Modulation of the Expression of Proteins by Chronic Morphine Treatment in Rat Striatum

The expression of β -actin protein was not modified in this treatment (Figure 2); therefore, this protein was used as a control of loading for each lane. As expected, the previously observed increase or decrease in mRNA levels previously observed reflect modifications of the corresponding proteins. Thus, as shown in Figure 2, Gfap and Arc immunoreactivity were increased in rats chronically treated with morphine as compared to control animals (1.77- and 2.33-fold, respectively). Moreover, while only a slight insignificant increase of dynamin mRNA level was observed, an upregulation of protein immunoreactivity was obtained (1.97-fold). In the case of tubulin, Tau, stathmin and Cltc, the downregulation was confirmed at the protein level (1.36-, 1.66-, 1.3-, and 1.43-fold, respectively). Surprisingly, the regulation of Gap43 mRNA and protein were not in agreement. While a significant decrease of the mRNA level was observed, the protein expression was significantly upregulated (1.3-fold).

DISCUSSION

The aim of this study was to examine the effects of chronic morphine treatment on gene expression for neuronal and glial cytoskeleton components, using quantitative real-time RT-polymerase chain reaction and immunoblot. The striatum was selected owing to its high level of μ opioid receptors and its role in morphine-induced behavioral sensitization involved in the establishment of an addiction state (Maisonneuve *et al*, 1992). Moreover, it has been shown that morphine administration increases the activity of dopaminergic neurons in substantia nigra pars compacta and increases dopamine release in the striatum (Walker *et al*, 1987; Pozzi *et al*, 1995). Samples were collected 16 h following the last morphine injection. It has been shown

Figure 1 Effect of chronic treatment (5 days) with morphine on the mRNA level of cytoskeletal genes by real-time RT-PCR analysis. All data have been normalized for levels of *Tbp* expression within the same sample and represent mean $\pm$ SD ($n = 6-8$ rats), * $P < 0.05$, ** $P < 0.01$ (Student's test).

that with this protocol the behavioral signs of spontaneous withdrawal appear 24 h after the last injection and the maximum severity of abstinence appears 36 h after the last dose of morphine (Ruiz *et al*, 1996). Moreover, the modifications reported, at the biochemical level, after spontaneous withdrawal from chronic morphine treatment are observed 24 h after the last morphine injection (Bassareo *et al*, 1995; Crippens and Robinson, 1994; Diana

et al, 1995). The modulation of protein expression observed here is thus more likely to represent an adaptation of the neuronal cytoskeleton to chronic morphine treatment, but we cannot exclude a contribution by the mild withdrawal experienced at this time point by the animals.

In accordance with previous results (Noble *et al*, 2000), we observed that an upregulation of dynamin protein expression and a slight but insignificant increase of

Figure 2 Effect of chronic treatment with morphine on the expression level of the tested proteins by immunoblot analysis. Columns are means $\pm$ SD ($n = 5-6$ rats) and expressed as percentage of saline treated rats. Representative immunoblots are presented above each graph. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's test).

dynamin mRNA was induced by chronic morphine treatment. Dynamin forms rings around the necks of clathrin-coated pits and is required for the excision of the vesicle from the plasma membrane. This process is important for receptor recycling (review in Sever, 2002). Interestingly, the expression level of clathrin heavy chain was found to be downregulated both at the mRNA and protein levels. The concomitant increase of dynamin and decrease of clathrin expression may be explained by the recent results from Iversen *et al* (2003) showing that a downregulation of clathrin induced an upregulation of dynamin in fibroblasts. These modulations resulted in an accumulation of clathrin-coated pits at the cell surface connected to the plasma membrane via long dynamin-wrapped necks (Iversen *et al*, 2003). Taken together, these results suggest that chronic morphine treatment alters membrane and probably receptors trafficking in rat striatum.

Arc (activity-regulated cytoskeleton-associated protein) is classified as an immediate-early gene because of its rapid

induction after stimulation. Chronic morphine produced a significant increase in the mRNA and protein levels of *Arc* in rat striatum. The modulation of this protein in different rat brain structures by several drugs of abuse has been previously described (Ammon *et al*, 2003; Fosnaugh *et al*, 1995; Freeman *et al*, 2002; Kodama *et al*, 1998; Tan *et al*, 2000). The exact function of *Arc* is unknown but its induction and localization in dendrites and soma (Steward and Worley, 2001; Wallace *et al*, 1998) led to the hypothesis of its involvement in cytoskeletal rearrangements during the process of synaptic plasticity (Lyford *et al*, 1995). Since *Arc* mRNA has a short half-life (Steward and Worley, 2001), the upregulation observed here is unlikely to result from a direct action of morphine, but rather from a long-term adaptation of the dendritic network. This supports the modification of dendritic branching observed following chronic morphine treatment (Robinson and Kolb, 1999).

Microtubules are cytoskeletal polymers of α/β tubulin heterodimers. They contribute to the formation, main-

tenance, and function of axons and dendrites (review in Signor and Scholey, 2000). Expression of regulators of microtubule dynamics in rat striatum was found to be modified by chronic morphine treatment. Expression levels of α -tubulin and Tau were significantly decreased both at the mRNA and protein levels. Tubulin polymerization is a highly dynamic process, implying that microtubules are constantly undergoing rapid transitions between growth to shrinkage states ('dynamic instability') (Mitchison and Kirschner, 1984). The dynamics and organization of microtubules are under the control of microtubule-associated proteins (MAPs) (review in Sayas *et al*, 2002). Tau proteins belong to the MAPs family, and favor the nucleation of new microtubules, reduce their dynamic instability, and stabilize them against depolymerizing agents (review in Billingsley and Kincaid, 1997). *In vitro*, the expression level of Tau proteins are decreased in the neuroblastoma cell line SH-SY5Y after 48 h treatment with morphine (Lew, 1997). This study presents the first evidence of Tau regulation by morphine *in vivo*. Conversely, microtubules can be destabilized by proteins such as stathmin (review in Cassimeris, 2002). The critical role of stathmin in the maintenance of axonal integrity has been demonstrated in stathmin-deficient mice (Liedtke *et al*, 2002). The downregulation of stathmin observed here represents the first report of its modulation by chronic morphine treatment *in vivo*. Taken together with the decrease in tubulin expression and the downregulation of a microtubule stabilizing factor (Tau), the decrease in stathmin expression suggests a disorganization of axonal cytoskeleton.

Another protein important for axonal elongation and plasticity is GAP-43. In this study, the expression of Gap43 was decreased at the mRNA level but increased at the protein level. This apparent discrepancy could be explained by the delay between mRNA modulation and protein regulation reported for this protein (Van der Zee *et al*, 1989). Gap43 is synthesized during outgrowth and regeneration at an increased rate and enriched in nerve growth cones (review in Skene, 1989). The upregulation of the protein observed here suggests enhanced sprouting activity after chronic morphine treatment.

Gfap is exclusively expressed in glial cells, where it represents the major intermediate filament protein (Eng and Ghirnikar, 1994). In this study, we observed an upregulation of Gfap both at the mRNA and the protein levels. An increase of Gfap in response to morphine in different brain regions has been reported by others: ventral tegmental area (Beitner-Johnson *et al*, 1993), spinal cord, and hippocampus (Song and Zhao, 2001). This enhanced expression of Gfap is also observed in the hippocampus of chronic heroin abusers (Buttner *et al*, 2000). An increase in Gfap is associated with astrogliosis in response to brain injury and is used as an indirect measure of neurotoxicity (review in Eng *et al*, 2000). Song and Zhao (2001) showed that the increase observed in Gfap levels after chronic morphine treatment could be attributed mainly to hypertrophy of astroglial cells. Moreover, they showed that glial cells are involved in morphine tolerance to analgesia. The increase of Gfap observed here confirms that this protein could be a marker of changes in neuron-glia signalling (Song and Zhao, 2001).

There is an increasing evidence that the expression and activity of cellular proteins are modified by chronic morphine administration. Adaptations in G proteins and cAMP system in numerous brain regions known to be involved in the classic effects of opiates, including the striatum, have been described (Kaewsuk *et al*, 2001; Matsuoka *et al*, 1994; Self *et al*, 1995; Selley *et al*, 1997). The modulation of transcription factors (CREB, Δ FosB) after chronic morphine treatment has been described (Lane Ladd *et al*, 1997; Maldonado *et al*, 1996). Several heat-shock proteins (hsp70, hsp40, hsp27 and hsp105) are upregulated in the frontal cortex of rats chronically treated with morphine (Ammon *et al*, 2003; Fan *et al*, 2002, 2003; Loguinov *et al*, 2001; Nestler *et al*, 2001). Our results showing an increase in the mRNA level of Arc and no change in that of actin are consistent with the results of Ammon *et al*. The present results also confirm the increase in dynamin protein level previously showed in the striatum of mice chronically treated with morphine (Noble *et al*, 2000).

The modifications of mRNA and proteins levels observed in this study are summarized in Table 2. We found significant regulation of several genes related to neuronal plasticity following chronic morphine treatment. Although we cannot exclude a contribution of mild withdrawal in the observed modifications, the differential expression of cytoskeletal genes supports and extends the previous studies showing morphological alteration of neurons (Skair-Tavron *et al*, 1996) after chronic morphine treatment. Moreover, chronic opiate exposure decreases neurofilament proteins in rat and human brain (Beitner-Johnson *et al*, 1992; Beitner-Johnson and Nestler, 1993), which are not only implicated in axonal transport but are also responsible for maintaining the caliber of axons (review in Al-Chalabi and Miller, 2003). In addition to this, the altered regulation observed in this study suggests membrane trafficking impairment, microtubule disorganization, neuritogenesis, and dendritic activation following chronic morphine treatment. These alterations of nerve functioning could contribute to the modification of synaptic transmission and thus underlie neuroadaptations to chronic morphine administration and the associated long-lasting behavioral sensitization.

Table 2 Summary Table Showing the Modifications of mRNA and Protein Levels in the Striatum of Rats Chronically Treated with Morphine as Compared to Control Group

Gene	mRNA	Protein
Arc	↗ (1.48*)	↗ (2.33**)
Gap43	↘ (0.7**)	↗ (1.3**)
α -Tubulin	↘ (0.64*)	↘ (0.73**)
Stathmin	↘ (0.65**)	↘ (0.77**)
Cltc	↘ (0.71*)	↘ (0.7***)
Dynamin-1	↔	↗ (1.97**)
Gfap	↗ (1.41*)	↗ (1.77**)
Tau	↘ (0.7**)	↘ (0.6**)

The numbers in parentheses represent the fold change. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's test).

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