0026-895X/96/040636-10$3.00/0

MOLECULAR PHARMACOLOGY, 49:636–645 (1996).

Induction of Chronic Fos-Related Antigens in Rat Brain by Chronic Morphine Administration

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Received August 25, 1995; Accepted January 10, 1995

SUMMARY

Previous studies have shown that repeated exposure to cocaine or to several other stimuli induces novel 35–37 kDa Fos-related antigens (chronic Fras) in specific brain regions. Induction of these proteins is associated with prolonged increases in AP-1 DNA binding activity that parallel the long half-life of the chronic Fras in brain. In the current study, we characterized regulation of the chronic Fras in response to prolonged exposure to morphine. After 5 days of morphine treatment, we observed increased levels of the chronic Fras and of AP-1 binding activity in rat striatum and nucleus accumbens, effects that were not seen in most other brain regions that we studied. Concomitant administration of naltrexone, an opioid receptor antagonist, prevented the induction of these proteins. Two-dimensional gel analysis showed that the chronic Fras induced by chronic morphine administration are identical to those induced after chronic cocaine and other treatments. A time course study indicated that chronic Fra induction was first apparent after 3 days of morphine treatment and peaked between 5 and 7 days of treatment in both the striatum and nucleus accumbens. Withdrawal studies demonstrated robust induction of several known acute Fras, including c-Fos, FosB, Fra-1, Fra-2, and ΔFosB, at 6 hr after naltrexone precipitation of withdrawal in the striatum, nucleus accumbens, and several other brain regions. Levels of these proteins returned to basal values by 72 hr. In contrast, no further induction of the chronic Fras was evident after 6 hr of withdrawal in the striatum and nucleus accumbens, but levels of the proteins increased beyond their already elevated chronic morphine values after longer periods (72 hr) of withdrawal, even though physical withdrawal symptoms had resolved at this time point. Chronic Fras were also induced after these prolonged withdrawal periods in several other brain regions, where the proteins were not induced by chronic morphine alone. We discuss the possible roles played by the chronic Fras in the adaptive responses of the brain to chronic opiate exposure and opiate withdrawal.

Although many of the primary pharmacological, electrophysiological, and behavioral consequences of acute morphine and cocaine administration are distinct (1, 2), the drugs share certain locomotor activating and reinforcing properties (3–5). A large behavioral and pharmacological literature has demonstrated the importance of the striatum and its ventral extension, the nucleus accumbens, in mediating these common responses. These brain regions also exhibit persistent biochemical changes in specific signaling proteins after long term exposure to morphine or other drugs of abuse, which are believed to mediate long-lasting changes in locomotor activity and reinforcing mechanisms associated with drug addiction (5–8). It has been hypothesized that these biochemical adaptations are mediated, at least in part, by long term changes in neuronal gene expression.

This work was supported by United States Public Health Service Grants DA07359, DA08227, and DA00203 and by the Abraham Ribicoff Research Facilities of the Connecticut Mental Health Center, State of Connecticut Department of Mental Health.

Transcription factors, which can enhance or repress expression of a given gene, are plausible targets of drug treatments. c-Fos, which is the product of an immediate early gene, dimerizes with a Jun family protein to form the AP-1 complex (9, 10). The complex then binds to AP-1 elements in the promoter regions of specific genes to affect transcription. An increasing number of neural genes (e.g., tyrosine hydroxylase, D_1 dopamine receptor, *N*-methyl-D-aspartate R_1 glutamate receptor, protein kinase A subunits, neurofilaments, neurotensin) have been shown to contain AP-1 elements, although their precise role in the regulation of these genes remains unclear.

Several laboratories have shown that acute administration of cocaine or other stimulants can induce AP-1 binding, as well as c-Fos and other acute Fras, including FosB, Fra-1, Fra-2, and ΔFosB, in the striatum and nucleus accumbens (11–17). In contrast, chronic administration of cocaine desensitizes the induction of these various acute Fras (14, 18) and

ABBREVIATIONS: AP-1, activator protein-1; Fra, Fos-related antigen; SC, subcutaneous or subcutaneously; EMSA, electrophoretic mobility shift assay; SDS, sodium dodecyl sulfate; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

instead induces apparently novel Fos-like proteins of 35–37 kDa, termed chronic Fras (19). The chronic Fras persist long after the drug treatment has ceased and show altered AP-1 binding properties compared with their acute Fos counterparts (19, 20). Chronic Fras are also induced in the brain in a region-specific manner by other chronic treatments (see Discussion). The chronic Fras may thereby mediate some of the unique long term effects of cocaine and other repeated treatments on gene expression.

Acute administration of morphine has also been reported to induce c-Fos in the striatum (21–23). This effect may be mediated by dopamine: morphine, acting primarily via μ opioid receptors (24), is reported to increase dopaminergic transmission to the striatum (e.g., Refs. 2, 8, and 25–27), and the striatal induction of c-Fos by morphine can be blocked by the administration of a D_1 dopamine receptor antagonist (23). However, the effects of chronic morphine treatment on Fos family proteins has not been investigated.

In the current study, we demonstrate that chronic administration of morphine induces the chronic Fras in the striatum and nucleus accumbens, with limited induction and some decreases seen in several other brain regions examined. We also characterized the regulation of these proteins during short and long term stages of antagonist-precipitated opiate withdrawal. Induction of the chronic Fras could mediate some of the long term effects that morphine exerts in specific brain regions after chronic exposure and during withdrawal.

Materials and Methods

Drug treatments. Male Sprague-Dawley rats (initial weight, 150–200 g; Camm Research Institute, Wayne, NJ) were used in the study. Rats were housed three or four to a cage with food and water available *ad libitum* under a 12-hr light/dark cycle. For the acute morphine experiments, animals were administered morphine sulfate (10 or 20 mg/kg SC; National Institute on Drug Abuse) and were killed 1, 1.5, 2, or 3 hr later. Control animals received an equal volume of physiological saline. All animals were handled and injected with saline SC daily for 4 days before the morphine or saline challenge to habituate the animals to the stress associated with the injections.

For the chronic morphine experiments, one morphine pellet (containing 75 mg of morphine base; National Institute on Drug Abuse) was implanted SC with the animals under light halothane anesthesia daily for 1–7 days, with 5 days serving as the standard regimen. This pelleting method provides continuous exposure to morphine and has been shown to induce both tolerance and dependence as measured behaviorally, electrophysiologically, and biochemically (28, 29). Concomitant treatment with naltrexone HCl (100 mg/kg; Sigma Chemical Co., St. Louis, MO) was accomplished as follows: the drug was administered via a SC injection (50 mg/kg) in a time-released oil suspension and an additional intraperitoneal injection (50 mg/kg) each day immediately before the implantation of the morphine pellet. This paradigm has been shown to block the development of morphine tolerance and dependence in rats based on behavioral, electrophysiological, and biochemical measures (29, 30). Control animals in the chronic studies underwent identical surgery but received no pellet implantations. In the naltrexone experiments, some animals received naltrexone alone in addition to undergoing sham surgery. All animals were killed 18 hr after the final pellet implantation. In some experiments, morphine sulfate (National Institute on Drug Abuse) was administered intermittently at escalating doses by repeated twice-daily SC injections for 10 days: days 1 and 2, 10 mg/kg; days 3 and 4, 20 mg/kg; days 5 and 6, 40 mg/kg; days 7 and 8, 80 mg/kg; and days 9 and 10, 120 mg/kg. Control rats received repeated saline injections. Rats were used 18 hr after the last injection. This treatment regimen has been shown to induce sensitization to the acute locomotor activating effects of morphine (27, 31).

For the precipitated opiate withdrawal experiments, rats were implanted with morphine pellets daily for 5 days. On day 6, the two most recent morphine pellets were removed with the animals under light halothane anesthesia. After a 1-hr recovery period, animals were treated with naltrexone HCl (100 mg/kg SC). Additional injections of naltrexone (at the same dose) were repeated 6 and 24 hr later. Previous studies have established that this dose and treatment regimen of naltrexone are required to produce maximal, sustained levels of opiate withdrawal for a period of 72 hr, at which time overt signs of opiate withdrawal are completely resolved (29). As a control, some animals received naltrexone injections without prior morphine treatment.

Brain dissections and tissue preparation. Brains were removed from decapitated rats and chilled in ice-cold physiological buffer (14). Frontal cortex was removed by gross dissection. The striatum, nucleus accumbens, and amygdala were excised from 1-mm-thick coronal brain slices with the use of a 12-gauge syringe needle as described previously (32). The locus ceruleus, ventral tegmental area, and substantia nigra were excised with use of a 15-gauge syringe needle. Bilateral punches of each region were pooled. Frontal cortex and lateral septal nuclei were obtained by gross dissection. In some experiments, anterior and posterior striata were excised from adjacent 1-mm coronal slices. In initial studies, striatum was obtained by gross dissection, which yielded the same results as the punch dissections.

Tissue was immediately Dounce homogenized in ~20× volumes of EMSA buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 20% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 10 μg/ml leupeptin, 0.1 mM *p*-aminobenzamidine, 1 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol) (see Ref. 14). Samples were then incubated for 30 min at 4° and centrifuged at 15,000 × *g* for 25–30 min. Resulting supernatants were aliquoted and frozen for subsequent analysis. Protein concentrations were determined according to the Lowry or Bio-Rad Bradford method.

One- and two-dimensional electrophoresis and Western blotting. SDS stop solution (final concentration, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) was added to aliquots of EMSA extracts containing 20–50 μg of total protein. Samples were then boiled and subjected to one-dimensional SDS-polyacrylamide gel electrophoresis at 60–70 V for 12–18 hr with 10% acrylamide/0.4% *N,N'*-methylenebisacrylamide resolving gels. For two-dimensional analysis, aliquots of EMSA extracts (containing 150–180 μg of protein) were separated by isoelectric focusing in tube gels for the first dimension according to published procedures (33). The resulting tube gels were then layered across SDS-polyacrylamide slab gels (9% acrylamide/0.36% bisacrylamide) and electrophoresed in the second dimension. In both cases, proteins in resulting gels were transferred electrophoretically onto nitrocellulose, blocked with 2% nonfat dry milk, and incubated with an anti-M-peptide (anti-Fra) primary antibody (1:4000; Ref. 12) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4000). Immunoreactivity was visualized by chemiluminescence. The specificity of the resulting immunoreactive bands was confirmed by demonstrating that the binding was abolished when the anti-Fra antibody was preabsorbed with purified M-peptide (Fig. 1A), as established previously (19, 20). Immunoblotting signals were quantified with computerized densitometry. For most experiments, statistical significance was calculated by use of the Student's *t* test. For some experiments, it was necessary to use the χ^2 test to compare data obtained on different days when control values can vary considerably.

Gel shift assay. The gel shift assay was performed exactly as described previously (14, 19). Briefly, the AP-1 probe used is a double-stranded synthetic oligonucleotide derived from the promoter sequence of the HMT II gene, identical to that used by Sonnenberg *et al.* (34). The probe was labeled with the Klenow DNA polymerase

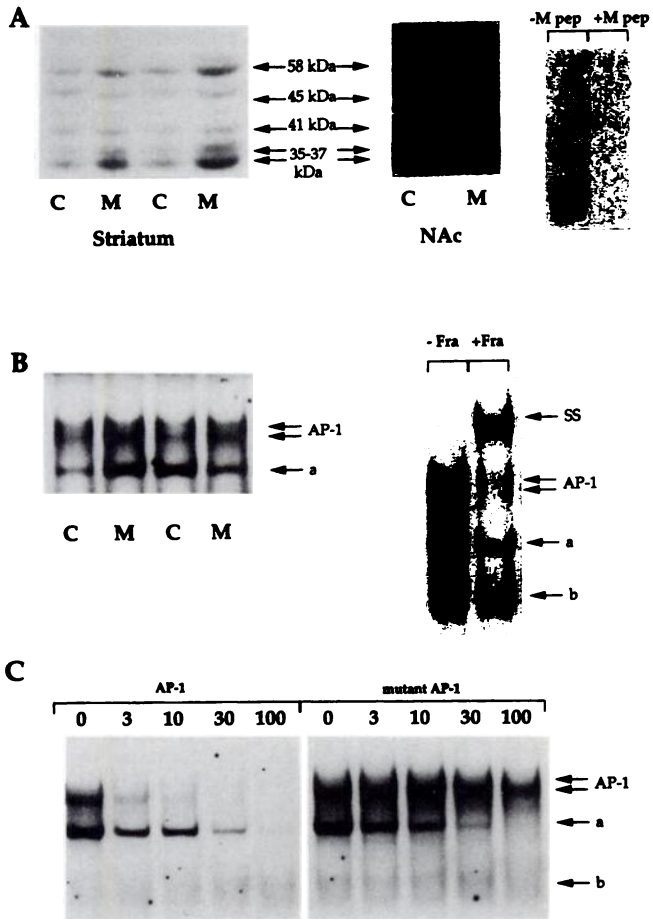

Fig. 1. Fos-like immunoreactivity and AP-1 binding in the striatum and nucleus accumbens (*NAc*) after chronic morphine administration. Animals received SC implantation of morphine pellets (75 mg) daily for 5 days (*M*) and were killed on day 6. *C*, control animals. Extracts of striatum (A, *left*) and nucleus accumbens (A, *middle*) were then analyzed for Fos-like immunoreactivity with Western blotting (see Materials and Methods). Striatum was also analyzed (B, *left*) for AP-1 DNA binding activity with gel shift assays (see Materials and Methods). A, *Arrows*, Fos-like immunoreactivity at 35–37 kDa (chronic Fras), 41 kDa (presumably Fra-1/Fra-2), 45 kDa (FosB), and 58 kDa (c-Fos). Specificity of the Fra antibody was demonstrated by preabsorption with purified M-peptide (A, *right*; see Materials and Methods). B, *Arrows*, doublet that represents specific AP-1 binding, as well as *bands a and b*, which represent nonspecific binding, based on Fra supershift (*SS*) (B, *right*; see Materials and Methods) and cold competition (C; see Materials and Methods) experiments. The results shown are representative of data obtained from analysis of 12 rats from two separate experiments for the striatum and 6 rats from one experiment for the nucleus accumbens.

fill-in reaction, with [α-^{32}P]dGTP and [α-^{32}P] dTTP to a specific activity of 2–9 $\times$ 10^8 cpm/μg. The probe was incubated with aliquots of EMSA extracts (containing 10–40 μg of protein) in binding buffer for 20 min at room temperature. The samples were then electrophoresed at 150 V for 3 hr in a nondenaturing 6% acrylamide/0.24% *N,N'*-methylenebisacrylamide gel containing 25 mM Tris borate, pH 8.3, 1 mM EDTA, and 1.6% glycerol. Resulting gels were dried and exposed to x-ray film. Bands were shown to be specific for AP-1 binding by cold competition and supershift assays as reported previously (19). Briefly, in the cold competition experiments, increasing concentrations (0, 3, 10, 30, and 100 ng) of unlabeled wild-type HMT AP-1 probe or mutant HMT AP-1 probe were added to samples to compete with the labeled AP-1 probe for specific or nonspecific binding proteins. Signals readily competed out by the wild-type AP-1 sequence and not by the mutant sequence were considered specific (Fig. 1C). Supershift experiments involved incubation of tissue extracts with 0.2 μl of anti-Fra antibody for 1 hr before the addition of labeled AP-1 probe and the gel shift analysis (Fig. 1B).

Results

Acute morphine regulation of Fos/Fra immunoreactivity in the striatum and nucleus accumbens. Previous reports have demonstrated that c-Fos is induced in the striatum at the protein and mRNA levels by immunohistochemical and Northern blotting techniques (21–23). As a first step in assessing the effects of morphine treatments on Fos-like proteins, we studied the effects of acute morphine administration on levels of c-Fos and other Fos family members in the striatum and nucleus accumbens by Western blotting. Two doses of morphine sulfate, administered SC, were used: 10 and 20 mg/kg. No Fos-like protein was significantly and consistently induced in either the anterior or posterior striatum or in the nucleus accumbens at 1, 1.5, or 2 hr after the acute challenge (data not shown). After 3 hr, however, a small induction of c-Fos (migrating at 58 kDa) was apparent in striatum and nucleus accumbens (Fig. 2). This induction was observed at both doses of morphine; however, the 10 mg/kg dose elicited a 2-fold greater increase in the nucleus accumbens compared with the 20 mg/kg dose; the two doses elicited increases of similar magnitude in the striatum (Fig. 2, *bottom*). In contrast to c-Fos, acute morphine administration (at either dose) did not alter levels of other Fos-like proteins in the striatum or nucleus accumbens at any of the time points examined, with the exception of a small induction in the nucleus accumbens of a 33-kDa protein (presumably ΔFosB) at 3 hr (Fig. 2).

Chronic morphine regulation of Fos/Fra immunoreactivity and AP-1 binding in the striatum and nucleus accumbens. To assess the effect of chronic morphine administration on Fos/Fra proteins, animals received a 5-day pellet treatment regimen known to produce tolerance and dependence (see Materials and Methods). As shown in Fig. 1A and Table 1, the most dramatic effect of chronic morphine treatment in the striatum was an increase in levels of 35–37-kDa Fos-like proteins, termed chronic Fras. Induction of the chronic Fras was accompanied by a much smaller, but consistent, increase in levels of higher molecular weight acute Fras, including c-Fos, FosB, and Fra-1/Fra-2. Chronic morphine treatment also led to a significant induction of the chronic Fras in the nucleus accumbens (Fig. 1A and Table 1), although there was no consistent effect on the acute Fras in this brain region.

Chronic morphine induction of the chronic Fras in the striatum and nucleus accumbens was associated with an increase in AP-1 binding activity in these brain regions. As shown for striatum in Fig. 1B, specific AP-1 binding activity was visualized as a tightly spaced doublet. We have previously shown that the lower, faster migrating band is associated with induction of chronic Fras, whereas the higher, slower migrating band is associated with induction of acute Fras in response to cocaine and other treatments (19, 20). Induction of both AP-1 bands in striatum by chronic morphine treatment is consistent with the induction of both chronic and acute Fras in this region as determined by Western blot analysis (Fig. 1A).

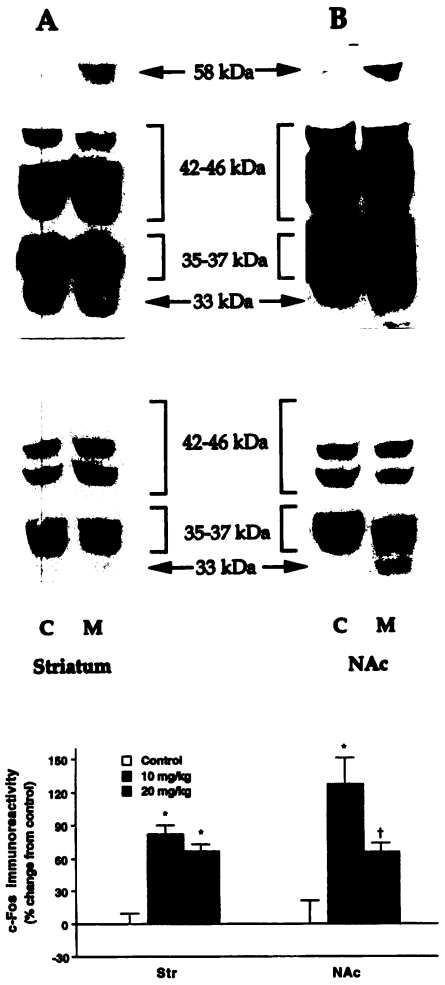

Fig. 2. Fos-like immunoreactivity in striatum (A) and nucleus accumbens (*NAc*, B) after acute morphine administration. Animals were given a single dose (10 or 20 mg/kg SC) of morphine sulfate (*M*) and killed 3 hr later. Control animals (*C*) received equivalent saline injections. Extracts of both regions were analyzed for Fos-like immunoreactivity with Western blotting, as described in Materials and Methods. *Top*, darker autoradiogram exposures (10 mg/kg dose); *bottom*, lighter autoradiogram exposures (10 mg/kg dose). There was a relatively small increase in c-Fos immunoreactivity (*arrow* at 58 kDa) after the acute morphine challenge in both regions. No changes in levels of other Fos-like proteins were observed except for a 33-kDa (ΔFosB), which was slightly increased in in the nucleus accumbens. All bands were shown to be specific by preabsorption of the antibody with purified M-peptide (see legend to Fig. 1). Data for c-Fos (*bottom*) are expressed as mean percent change from control ± standard error (four animals in each treatment group). †, $p < 0.05$, *, $p = \sim 0.6$, by *t* test compared with control.

TABLE 1

Regional specificity of chronic Fra induction by chronic morphine treatment

Animals received sc implantation of morphine pellets (75 mg) daily for 5 days and were killed on day 6. Brain extracts were then analyzed for Fos-like immunoreactivity by Western blotting (see Materials and Methods). Values shown were obtained by densitometry measurements of the Western blot signals of the 35-kDa plus the 37-kDa chronic Fra bands. As a result, the values shown in the table do not as accurately reflect selective changes in the 37-kDa band that were observed in certain regions (i.e., locus ceruleus, substantia nigra, and ventral tegmental area) and depicted in Fig. 6.

Region	Chronic Fra Immunoreactivity
	% of control ± standard error (n)
Striatum	177 ± 33 (12)[a]
Nucleus accumbens	167 ± 12 (6)[a]
Amygdala	75 ± 26 (6)
Frontal cortex	108 ± 5 (8)
Lateral septal nucleus	79 ± 12 (6)
Locus ceruleus	71 ± 9 (4)
Substantia nigra	127 ± 12 (4)
Ventral tegmental area	108 ± 7 (4)

[a] $p < 0.05$ by *t* test.

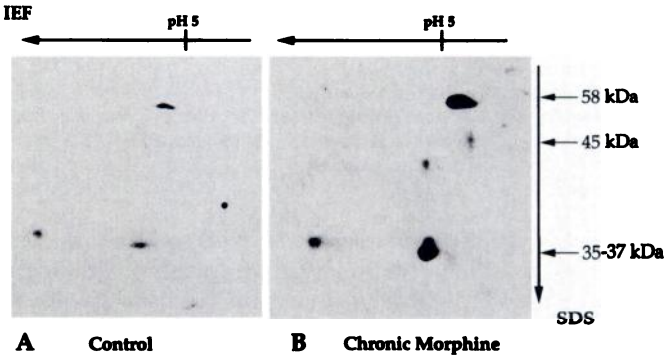

Fig. 3. Two-dimensional analysis of Fos-like proteins induced in the striatum by chronic morphine administration. Extracts of striatum from control (A) and chronic-morphine treated (B) rats used for one-dimensional Western blotting (see legend to Fig. 1) were also analyzed with two-dimensional Western blotting. The first dimension separated proteins by isoelectric focusing (*IEF*): *left*, basic end; *right*, acidic end. pH range is indicated above each resulting gel. In the second dimension, proteins were further resolved by SDS-polyacrylamide gel electrophoresis. *Right*, apparent molecular mass. B, *Arrows*, Fos-like proteins induced by chronic morphine treatment: 35–37 kDa (chronic Fras), 41 kDa (presumably Fra-1/Fra-2), 45 kDa (FosB), and 58 kDa (c-Fos). The results shown are representative of data obtained from analysis of four animals in each treatment group.

Characterization of Fos/Fra induced by chronic morphine treatment by two-dimensional Western blotting. Verification that the 35–37-kDa chronic Fras induced in the striatum by chronic morphine treatment are identical to those induced by chronic cocaine and other treatments was achieved by two-dimensional electrophoretic analysis (Fig. 3). Striatum extracts from control and chronic morphine-treated animals were subjected to two-dimensional gel electrophoresis, and resulting gels were then Western blotted with the anti-Fra antibody to yield a two-dimensional pattern of Fos/Fra immunoreactivity. The 35–37-kDa bands induced by chronic morphine treatment comigrated precisely with the 35–37-kDa chronic Fras induced by chronic cocaine and other treatments (19, 20). Moreover, chronic morphine treatment induced additional bands of 58, 45, and 41 kDa, presumed to represent c-Fos, FosB, and Fra-1/Fra-2, respectively, which likely reflect the acute Fras whose induction was observed with one-dimensional Western blotting (Fig. 1A).

Naltrexone blocks the effects of chronic morphine treatment on Fos/Fra induction. Naltrexone, a specific and long-lasting opioid receptor antagonist, was administered concomitantly with the 5-day morphine pellet treatment to determine whether the morphine effect on Fras was specific, that is, mediated via the activation of opioid receptors. Immunoblotting results showed that the administration of naltrexone, under conditions that block the development of opiate tolerance and dependence (see Materials and Methods), completely blocked the induction of the chronic Fras in the striatum (Fig. 4). The low level of induction of several

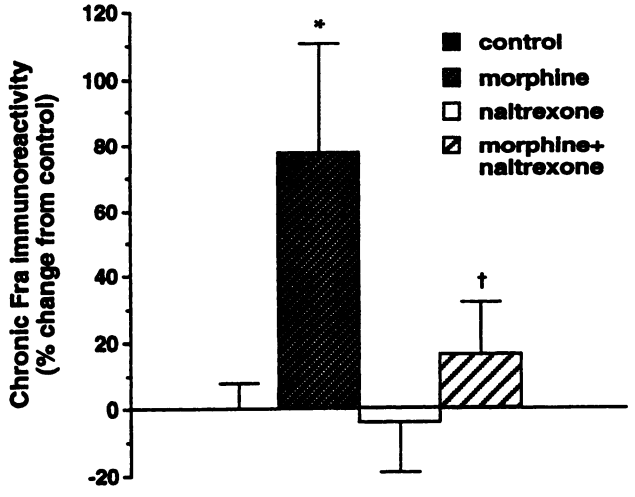

Fig. 4. Fos-like immunoreactivity in the striatum after concomitant morphine and naltrexone administration. Naltrexone was administered under conditions known to block the development of opiate tolerance and dependence, as described in Materials and Methods. Animals were treated with morphine alone, naltrexone alone, or both drugs concomitantly. Control rats received sham surgery and vehicle injections. Striatum extracts were then analyzed for Fos-like immunoreactivity by Western blotting (see Materials and Methods). Data are expressed as mean percent change from control ± standard error (6 animals for the naltrexone and naltrexone plus morphine conditions and 12 animals for the control and morphine conditions). *, $p < 0.05$ by t test compared with control. †, $p < 0.05$ by χ^2 test compared with morphine group.

acute Fras (e.g., see Fig. 1A) was also blocked by concomitant naltrexone treatment (data not shown). In contrast, chronic administration of naltrexone alone did not have any appreciable effect on levels of the chronic or acute Fras (Fig. 4).

Time course of Fos/Fra induction by chronic morphine treatment. To more precisely define the temporal features of chronic morphine regulation of Fos/Fra induction, we administered morphine pellets daily for 1, 3, or 7 days to bracket the standard chronic treatment period of 5 days. All animals were used 18 hr after the last pellet implantation. The Western blots shown in Fig. 5A demonstrate that levels of the chronic Fras are increased in the striatum after the 3- and 7-day treatment periods; the degree of increase seen after 7 days of treatment was comparable to that observed after 5 days (Fig. 1). In contrast, no increase was observed after the 1-day treatment. A similar time course of chronic Fra induction was seen in the nucleus accumbens (Fig. 5B). Induction of the acute Fras exhibited a more complex time course. In the striatum, levels of acute Fras were decreased after 1 day of treatment, unchanged after 3 days of treatment, and slightly increased after 7 days of treatment (Fig. 5A); this latter increase is similar to that seen after the 5-day treatment regimen (see Fig. 1A). However, comparison of Figs. 1A, 3, and 5A illustrate the interanimal variability observed for this small induction of acute Fras in the striatum by chronic morphine administration; in some animals (e.g., Fig. 1A), c-Fos (58 kDa) was the major acute Fra induced, whereas in others (e.g., Fig. 5A), FosB (45 kDa) was the major acute Fra induced, although darker exposures revealed the induction of both bands in all animals studied (e.g., Fig. 3). The basis for this interanimal variability in acute Fra regulation is not known but has also been observed in response to other stimuli such as cocaine (38). In the nucleus accumbens, there was no significant effect on levels of acute Fras at 1, 3, or 7 days of treatment (Fig. 5B), which is consistent with the lack of effect seen after the 5-day treatment regimen (Fig. 1B).

It was also of interest to determine whether chronic intermittent exposure to morphine results in chronic Fra induction, as found for the pellet treatment regimen, which provides continuous exposure to the drug. It was found that morphine, given by repeated SC injections (see Materials and Methods), significantly increased levels of the chronic Fras in the striatum (144 ± 12% of control ± standard error, seven animals; $p < 0.05$ by t test). Because intermittent treatment regimen results in sensitization to some of the acute effects of morphine (25, 31), whereas the continuous treatment regimen results primarily in tolerance (28), our results indicate that chronic morphine induction of the chronic Fras is not associated uniquely with either sensitization or tolerance.

Regional distribution of chronic Fra induction by chronic morphine treatment. We next studied the regional specificity of the induction of the chronic Fras by chronic morphine administration. The regions examined were selected based on the known regulation of c-Fos in these regions by acute morphine treatment or precipitated opiate withdrawal (see Discussion). Fig. 6 shows representative lanes of Western blots from each region examined. After 5

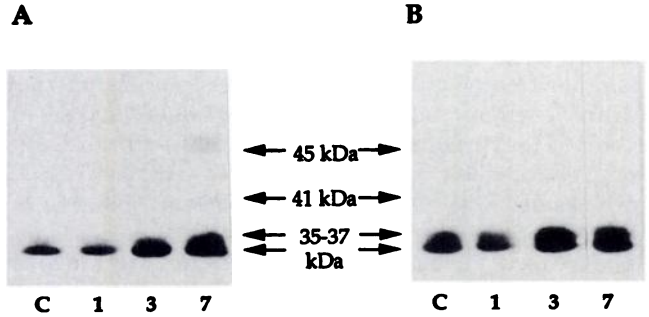

Fig. 5. Time course of induction of Fos-like immunoreactivity in the striatum (A) and nucleus accumbens (B) after chronic morphine administration. Animals received SC implantation of morphine pellets (75 mg) daily for 1, 3, or 7 days and were killed 18 hr after the last pellet implantation. *C*, control animals. Striatum and nucleus accumbens extracts were then analyzed for Fos-like immunoreactivity with Western blotting (see Materials and Methods). *Arrows*, positions of Fos-like proteins (see legends to Figs. 1 and 3). The results shown are representative of data obtained from the analysis of four animals in each treatment group.

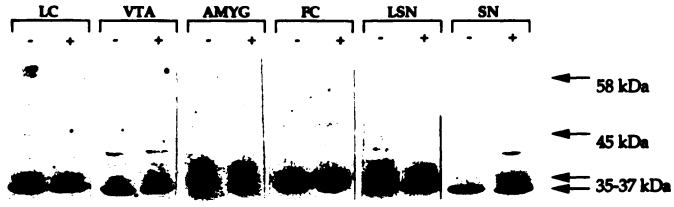

Fig. 6. Regional distribution of chronic Fra induction by chronic morphine administration. Animals received SC implantation of morphine pellets (75 mg) daily for 5 days and were killed on day 6. Extracts of locus ceruleus (*LC*), ventral tegmental area (*VTA*), amygdala (*AMYG*), frontal cortex (*FC*), lateral septal nucleus (*LSN*), and substantia nigra (*SN*) were analyzed for Fos-like immunoreactivity with Western blotting (see Materials and Methods). Different amounts of protein were analyzed for each region, so direct comparisons of basal levels of Fos-like immunoreactivity among the regions are not possible. The results shown are representative of data obtained from the analysis of four samples in each treatment group for locus ceruleus, ventral tegmental area, and substantia nigra (with each sample derived from two animals), six samples for amygdala and lateral septal nucleus, and seven samples for frontal cortex.

days of morphine pellet implantations, chronic Fra levels were not consistently changed in frontal cortex, amygdala, or lateral septal nucleus, although a decrease was evident in some amygdala and lateral septal nucleus samples. In contrast, increased levels of the 37-kDa chronic Fra band, in particular, were evident in the substantia nigra and ventral tegmental area. This increase was consistently present in the substantia nigra; it was observed in four of four samples analyzed, with each sample representing tissue pooled from two rats. In contrast, the increase was more variable in the ventral tegmental area; it was seen in two of four samples analyzed. This intersample variability most likely represents interanimal variability because our dissections are highly reproducible and because such interanimal variability in other biochemical measures in this brain region has been related to interanimal differences in behavior (35). Changes were also noted in the locus ceruleus, where there was a consistent decrease (observed in four of four samples analyzed) in the 37-kDa chronic Fra band (Fig. 6).

Table 1 provides numerical data for the effect of chronic morphine on chronic Fra levels. However, it is important to note that these data represent levels of both chronic Fra bands (i.e., the 35- and 37-kDa bands) and therefore does not depict selective regulation of the 37-kDa band as illustrated in Fig. 6 for the substantia nigra, locus ceruleus, and ventral tegmental area.

Accompanying the chronic morphine-induced decrease in chronic Fras in the locus ceruleus is a decrease in the basal level of c-Fos migrating at 58 kDa (Fig. 6). This finding is consistent with a previous study (22), which demonstrated decreased levels of c-Fos protein and mRNA in the locus ceruleus after chronic morphine treatment.

Induction of Fos/Fra immunoreactivity during naltrexone-precipitated opiate withdrawal. After the 5-day chronic morphine treatment regimen, physical opiate withdrawal was precipitated by administration of naltrexone on day 6. All animals demonstrated behaviors characteristic of opiate withdrawal, including jumping, wet dog shakes, teeth chatter, ptosis, lacrimation, diarrhea, and irritability. Animals were given additional naltrexone treatments 6 and 24 hr later, which are required to sustain a maximal level of withdrawal as demonstrated previously (29). Consistent with our earlier observations, high levels of withdrawal were apparent at 6 hr, less severe symptoms were apparent at 24 hr, and no detectable withdrawal behaviors were observed at 72 hr.

In one withdrawal experiment, we studied the effect of opiate withdrawal on levels of acute and chronic Fras in representative brain regions at the 6-, 24-, and 72-hr time points (Fig. 7A). In another experiment, we compared levels of acute and chronic Fras observed after 6 and 72 hr of withdrawal with those observed in the chronic morphine-treated state (without withdrawal) (Fig. 7C). After 6 hr of withdrawal, there was a dramatic increase in several acute Fras, as shown by Western blotting, including c-Fos, FosB, Fra-1/Fra-2, and ΔFosB, in the striatum and nucleus accumbens (Fig. 7, A and C). Levels of these proteins remained increased, although to a lesser extent, after 24 hr of withdrawal, and most returned to basal levels after 72 hr. Induction of FosB, however, persisted at the 72-hr time point, particularly in the nucleus accumbens. The chronic Fras showed a different time course during withdrawal in these brain regions. Levels of the chronic Fras were increased with chronic morphine (without withdrawal) (Fig. 7C), as seen before (e.g., Figs. 2 and 5). Chronic Fra levels were also increased at the 6-hr time point relative to naive controls (Fig. 7, A and C). These increases were similar in magnitude to those seen after chronic morphine alone and seem to

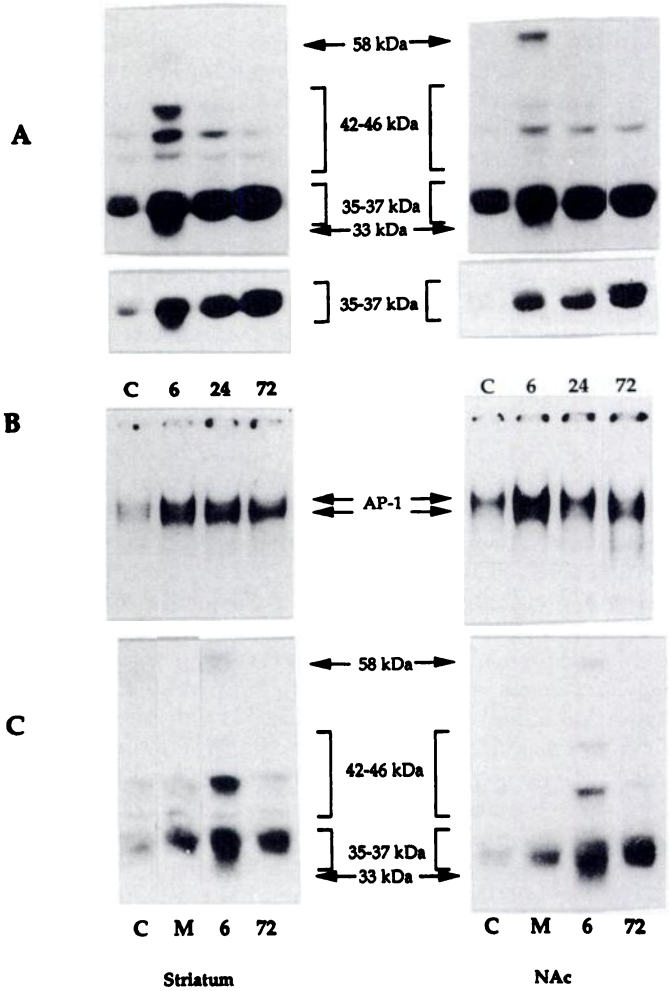

Fig. 7. Fos-like immunoreactivity and AP-1 binding in the striatum and nucleus accumbens (*NAc*) during naltrexone-precipitated opiate withdrawal. Animals received SC implantation of morphine pellets (75 mg) daily for 5 days (*M*). On day 6, they were given naltrexone (100 mg/kg intraperitoneally) to precipitate opiate withdrawal. Naltrexone injections were repeated after 6 and 24 hr of withdrawal. This naltrexone treatment regimen has been shown to result in maximal, sustained levels of withdrawal for a period of 72 hr, as described in Materials and Methods. A and B, Groups of animals were killed after 6, 24, and 72 hr of withdrawal. C, Groups of animals were killed with saline injections instead of naltrexone [i.e., chronic morphine (*Chr*)] or after 6 and 72 hr of withdrawal. In both experiments, naive rats served as controls (*C*). [In separate experiments (see text), naltrexone was shown to have no effect on acute or chronic Fras after acute or repeated administration.] Striatum and nucleus accumbens extracts were then analyzed for Fos-like immunoreactivity by Western blotting (A and C) and for AP-1 DNA binding activity by gel shift assay (B) (see Materials and Methods). *Arrows on Western blots*, positions of Fos-like proteins (see legend to Fig. 2). A, *Bottom*, lighter autoradiographic exposures of the chronic Fra region of the same blots depicted on the *top* to improve visualization of the chronic Fras. *Arrows on gel shift autoradiograms*, specific AP-1 binding complexes (see Materials and Methods). Results are representative of data obtained from the analysis of four animals in each treatment group.

reflect no additional effect of the 6-hr withdrawal per se. However, levels of the chronic Fras were increased further in the striatum and nucleus accumbens after longer periods of withdrawal, particularly evident at the 72-hr time point (Fig. 7, A and C). This suggests an additive effect of chronic morphine treatment and prolonged morphine withdrawal on induction of these proteins. As an additional control, we found that acute and repeated naltrexone administration to naive rats was not associated with induction of acute or chronic Fras in the striatum or nucleus accumbens (data not shown; see also Fig. 4).

AP-1 binding in the striatum and nucleus accumbens paralleled the Western blotting results. At 6 hr after initiation of withdrawal, a robust increase in AP-1 binding was observed in the striatum and nucleus accumbens (Fig. 7B). Increased AP-1 binding persisted through 24 and 72 hr of withdrawal, although not with the same magnitude as seen for the 6-hr time point. As shown in Fig. 7B, the increase in AP-1 binding seen at 6 hr of withdrawal was composed of both the top and bottom bands. This is consistent with elevated levels of both acute and chronic Fras at this time point. In contrast, the increase in AP-1 binding seen at 72 hr of withdrawal was composed predominantly of the bottom band, consistent with elevated levels of the chronic Fras only at this time point. Induction of both acute and chronic Fras after 6 hr of withdrawal presumably accounts for the greater induction of AP-1 activity observed at this early withdrawal time point.

We also studied Fos/Fra induction in the locus ceruleus, ventral tegmental area, and frontal cortex, where acute opiate withdrawal has been shown to increase levels of c-Fos protein or mRNA (22, 36, 37). Consistent with these earlier studies, levels of c-Fos immunoreactivity were strongly induced in all three brain regions after 6 hr of withdrawal (Fig. 8; frontal cortex data not shown). In addition, there were increases in the other acute Fras, as observed for the striatum and nucleus accumbens. Levels of Fos/Fra induction, however, were not equivalent among the regions studied. The responses seen in the locus ceruleus were more robust than those in either the ventral tegmental area or frontal cortex; these results correspond to the relative induction of c-fos mRNA levels in these regions during opiate withdrawal found in an earlier study (22). Furthermore, the nature of the banding pattern in the 40–46-kDa range in withdrawing animals showed considerable variation among the regions studied. Frontal cortex showed many species (tightly spaced bands) induced in this range, whereas locus ceruleus and ventral tegmental areas showed only a few sparsely spaced bands. These unique patterns of Fos-family proteins suggest region-specific differences in neuronal response and adaptation during opiate withdrawal. As observed in the striatum and nucleus accumbens, levels of induction of c-Fos and the other acute Fras declined by 24 hr and were close to basal values by 72 hr.

As shown in Fig. 8, the chronic Fras were not consistently altered in the locus ceruleus or ventral tegmental area after 6 hr of withdrawal. Similar results were obtained for the frontal cortex (data not shown). This is consistent with the lack of significant induction of these proteins in these regions after chronic morphine treatment (without withdrawal), as reported above (see Fig. 6). However, there was a small but consistent increase in the 35-kDa chronic Fra band after 24 and 72 hr of withdrawal and in the 37-kDa band after 72 hr of withdrawal in the locus ceruleus. Similar effects were seen in the ventral tegmental area, although the magnitude of chronic Fra induction was smaller than that observed in the locus ceruleus. No consistent induction of either chronic Fra band was seen in the frontal cortex at these later withdrawal time points.

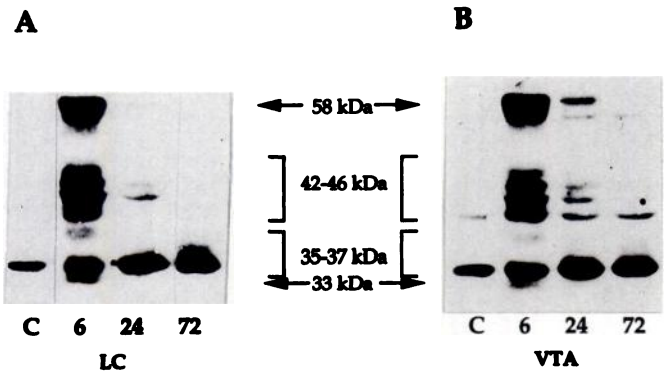

Fig. 8. Fos-like immunoreactivity in the locus ceruleus (*LC*) and ventral tegmental area (*VTA*) during naltrexone-precipitated opiate withdrawal. Animals received SC implantation of morphine pellets (75 mg) daily for 5 days. On day 6, they were given naltrexone (100 mg/kg intraperitoneally) to precipitate opiate withdrawal. *C*, control animals. Naltrexone injections were repeated after 6 and 24 hr of withdrawal. Groups of animals were killed after 6, 24, and 72 hr of withdrawal. Locus ceruleus (A) and ventral tegmental area (B) extracts were then analyzed for Fos-like immunoreactivity with Western blotting (see Materials and Methods). *Arrows*, positions of Fos-like proteins (see legend to Fig. 1). Results are representative of data obtained from the analysis of four samples in each treatment group (with each sample derived from two animals).

Discussion

The major finding of this study was that novel Fos-like proteins of 35 and 37 kDa, termed chronic Fras, are induced in the striatum and nucleus accumbens by chronic morphine administration. Induction of these proteins is associated with increases in levels of AP-1 DNA binding activity in these brain regions. Induction of the chronic Fras by morphine was time dependent and required chronic morphine administration. In addition, chronic Fra induction was blocked by concomitant administration of naltrexone, indicating that the effect is mediated via activation of opioid receptors, and was not observed in several other brain regions examined.

These findings add morphine to a growing number of treatments that have been shown to induce the chronic Fras in brain. In each case, induction of the proteins is observed uniquely in response to chronic, as opposed to acute, treatment paradigms and occurs in a region-specific manner. Thus, chronic Fra induction has been demonstrated in the striatum and nucleus accumbens after chronic cocaine treatment (19), in cerebral cortex and hippocampus after chronic electroconvulsive seizures (20), in frontal cortex after chronic antidepressant drug administration (19), in the striatum after chronic antipsychotic drug administration (38), in the striatum after 6-hydroxydopamine lesions of the substantia nigra with and without subsequent repeated injections of D_1 dopamine receptor agonists (19, 39), and in hippocampus after kainate lesions (40). Two-dimensional Western blotting has confirmed that the same chronic Fra proteins are induced after these various treatments (20), as we have dem-

onstrated for chronic morphine treatment in the present study. Although chronic Fra induction may be a general response of the brain to many types of chronic perturbations, it is important to emphasize that the specificity of the chronic Fra response resides in the fact that each treatment induces the proteins only in specific target brain regions. Within this context, it is of particular interest that the chronic Fras are induced most robustly in the striatum and nucleus accumbens after chronic morphine or cocaine administration as these brain regions are believed to be one site of convergence where these and other drugs of abuse produce locomotor sensitization and motivational aspects of drug addiction (e.g., drug craving) (see introductory paragraphs).

In addition to the chronic Fras, we observed low levels of regulation of acute Fras by acute and chronic morphine administration. A single, acute injection of morphine was found to elicit a small increase in c-Fos in both the striatum and nucleus accumbens, with no effect observed on other acute Fras (FosB, Fra-1, Fra-2, and ΔFosB) except for a small increase in ΔFosB in the nucleus accumbens. The small magnitude of this effect, relative to the 5–10-fold increases in c-Fos typically seen with many other acute stimuli (10), raises the possibility that the induction of c-Fos protein and mRNA by acute morphine observed previously with the use of immunohistochemistry and *in situ* hybridization (21–23) occurs in a small subset of neurons within each of these regions. The induction of c-Fos by acute morphine administration showed a nonlinear dose response. A similar nonlinear pattern has been observed for induction of c-Fos and other acute Fras by acute cocaine administration in the striatum and nucleus accumbens (38). Although the explanation for this nonlinear pattern remains unknown, it could reflect the recruitment of additional neural mechanisms by the higher doses of the drugs that serve to dampen Fos/Fra induction. After prolonged periods of continuous morphine treatment, levels of c-Fos and several other acute Fras in the striatum first show a small decrease from their low basal levels, followed by a sustained, albeit small, increase after longer treatment periods. In the nucleus accumbens, consistent changes in levels of acute Fras were not observed.

Morphine regulation of the acute Fras is very different compared with that observed for several other treatments. For example, as mentioned above, after a single acute injection of cocaine or a single electroconvulsive seizure, there is a dramatic induction of c-Fos and the other acute Fras, respectively, in the striatum and nucleus accumbens (11–17) and in cerebral cortex and hippocampus (34). After repeated cocaine or seizure administration, levels of the acute Fras are reduced below control values and exhibit muted (desensitized) increases in response to a subsequent cocaine or seizure challenge (14, 18, 41). The reasons for the differential regulation of the acute Fras by acute and chronic morphine exposure require further investigation.

We also studied the regulation of Fos/Fra induction during opiate withdrawal. As reported previously (22, 36, 37), c-Fos was induced in several brain regions after short periods (6 hr) of withdrawal. Other acute Fras, including FosB, Fra-1, Fra-2, and ΔFosB, were found to be similarly induced in these regions. Although levels of these acute Fras declined from 6 to 24 hr of withdrawal, they remained consistently elevated at 24 hr and returned to basal values only after 72 hr of withdrawal. In fact, FosB remained consistently elevated in some regions even at the 72-hr time point. This time course of the acute Fras during withdrawal represents a much more prolonged induction of these proteins compared with that observed after single, acute treatments (e.g., cocaine or seizure), after which levels revert to normal within 8–12 hr (12, 14, 19, 20, 34). The more prolonged induction during withdrawal presumably reflects the fact that sustained opiate withdrawal is very different from a single drug treatment, in that withdrawal is a dynamic process during which there is persistent activation of several neural systems. Indeed, locus ceruleus neurons, for example, remain persistently activated after 24 hr of withdrawal (29).

A different pattern of chronic Fra induction was observed during withdrawal. There was no apparent increase in chronic Fra levels after short (6 hr) periods of withdrawal, but significant induction was seen after 24 and 72 hr in several brain regions studied. It is paradoxical that the chronic Fras are induced in the striatum and nucleus accumbens by chronic morphine treatment and further induced in these regions after prolonged withdrawal. One possibility is that the chronic Fra induction observed under these two conditions occurs within different neuronal populations. Another possibility is that the induction occurs within the same cell types but is mediated via different mechanisms, e.g., via direct regulation of opioid receptors on striatum and nucleus accumbens neurons versus indirect regulation of various afferent pathways to these neurons. It is important to mention in this context that a similar paradox is observed for regulation of c-Fos in these same brain regions: c-Fos is induced by acute morphine treatment as well as by acute morphine withdrawal. Further studies are needed to investigate the validity of these and alternative interpretations of the data.

It is interesting that levels of the chronic Fras are maximally increased in the various brain regions studied after 72 hr of withdrawal because at that time, animals no longer show overt signs and symptoms of physical withdrawal (29). This is particularly striking for the locus ceruleus, which is known to play an important role in mediating physical opiate withdrawal (42–44). The firing rate of locus ceruleus neurons, although ~2-fold above control values after 24 hr of withdrawal, returns to normal after 72 hr (29). Moreover, up-regulation of the cAMP second messenger and protein phosphorylation system, which mediates part of the withdrawal activation of these neurons (44, 45), is also normalized after 72 hr of withdrawal (29). Thus, even when the locus ceruleus neurons are indistinguishable from basal conditions based on available electrophysiological and biochemical criteria and the withdrawal behaviors subserved by locus ceruleus neuronal activation have largely subsided, levels of the chronic Fras are still rising in these cells. These findings highlight that biochemical adaptations are occurring in the locus ceruleus and elsewhere long after the precipitation and gross resolution of withdrawal. Indeed, the chronic Fras could mediate some of the more subtle and protracted aspects of opiate withdrawal that have been documented in animals and people (46). In the locus ceruleus, persistent expression of the chronic Fras could mediate physical aspects of protracted withdrawal, whereas in the striatum and nucleus accumbens, their persistent expression could mediate some of the locomotor and motivational aspects of protracted withdrawal.

Although the precise role played by the chronic Fras in chronic morphine action and in prolonged morphine withdrawal remains unknown, characterization of the regulation of these novel proteins in specific brain regions provides clues as to the complex changes that are occurring in the brain under these conditions. Clearly, a better understanding of the functional role played by the chronic Fras requires identification of these proteins as well as of the genes influenced by them. Recent studies have shown that the chronic Fras can be distinguished from c-Fos, FosB, Fra-1, and Fra-2 but are immunochemically related to ΔFosB, a truncated splice variant of FosB (17, 19). However, they can be distinguished from ΔFosB based on the temporal properties of the proteins and on their migration on one- and two-dimensional gels. Therefore, the chronic Fras seem to be unique splice variants of the FosB or a related gene or unique post-translational modifications of FosB-like proteins. Further characterization of the proteins, which is currently under way, will be required to resolve this. Nevertheless, AP-1 complexes composed of the chronic Fras exhibit different DNA-binding properties than AP-1 complexes composed of the acute Fras (20). In addition, the chronic Fras, and the AP-1 complexes they form, exhibit much longer half-lives in brain compared with the acute Fras and their associated AP-1 complexes (19, 20). Thus, the chronic Fras represent attractive candidates as "molecular switches" that mediate some of the effects of chronic perturbations on the brain. Further study of these proteins promises to reveal novel transcriptional mechanisms by which chronic morphine and other treatments produce long-lasting effects in target brain regions.

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