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Precipitated Morphine Withdrawal Stimulates Multiple Activator Protein-1 Signaling Pathways in Rat Brain

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SUMMARY

Morphine dependence is a long lasting form of neuronal plasticity. Naloxone-precipitated morphine withdrawal, a model of opioid dependence, induces brain region-specific changes in activator protein-1 (AP-1) transcription factor gene expression. Rapid increases in c-*fos*, *fos*-B, *jun*-B, and c-*jun* mRNA levels accompany withdrawal, with the relative level of induction correlating with the severity of physical dependence. Altered patterns of c-*fos* mRNA expression were limited to neuronal circuits mediating stress responses, motivation, and cognition. AP-1 DNA-binding activity and dimer composition also exhibited regulation after withdrawal, presumably as a result of both transcriptional and post-translational events. Thus, morphine dependence results in the alteration of diverse, brain region-specific, signal transcription pathways involving AP-1 transcription factors.

Morphine withdrawal exposes the physical and motivational components of drug dependence and thus drug addiction. The severity of morphine dependence is related to the extent of morphine use, and its symptoms persist long after elimination of the drug from the body (1–4). Such long-lasting behavioral modifications hint of plastic changes within the nervous system, some of which may be partially mediated by regulation of gene expression (5, 6).

The idea of transcriptional regulation as a mediator of certain aspects of neural plasticity has been strengthened by studies on the regulation of expression of *fos* and *jun* genes (7, 8). These genes encode transcription factors that can regulate downstream gene expression by acting on target DNA sequences (referred to as the AP-1 DNA recognition sequence) near promoter elements (9). AP-1-mediated gene expression via *fos* and *jun* transcription factors involves several levels of control. First, Fos and Jun proteins bind their cognate DNA sequences as dimers, with specific combinations of Fos/Jun and Jun/Jun being predicted to dimerize *in vitro* (10). Second, dimer affinities for the AP-1 site vary (11). Third, protein phosphorylation states are critical for both basal and stimulated activity (12–14). Therefore, regulation of gene expression through the AP-1 enhancer element is more complex than merely increasing the transcriptional activity of *fos* and *jun* genes to elevate their mRNA, and consequently protein, levels.

Within the past few years, a variety of studies have shown a potentially important role for *fos* and *jun* gene expression, and subsequent AP-1 complex formation, in the neural plasticity associated with the administration of drugs of abuse. Morphine treatment, for example, has been shown to induce brain region-specific expression of the *fos* and *jun* genes. Acute morphine treatment induces a naloxone-sensitive increase in c-*fos* mRNA levels within the caudate putamen (15). Within the locus ceruleus, however, a decrease in basal c-*fos* mRNA levels follows acute morphine administration (16). Increased Fos immunoreactivity within the caudate putamen, as well as in the paraventricular nucleus and ventromedial nucleus of the hypothalamus, has also been reported after acute morphine administration (17). During morphine withdrawal, both c-*fos* and c-*jun* mRNA levels are increased within the locus ceruleus, a site believed to mediate some of the symptoms of morphine withdrawal. c-*fos* mRNA levels are also increased within the amygdala, ventral tegmentum, nucleus accumbens, neostriatum, and cerebral cortex after withdrawal (16).

To better characterize modulation of the AP-1 system after morphine administration, the effects of naloxone-precipitated morphine withdrawal were examined with regard to expression of *fos* and *jun* genes and AP-1 DNA/protein complex formation within various regions of the rat brain. Precipitated morphine withdrawal produced brain region-specific increases in the levels of a subset of *fos* and *jun* mRNAs, AP-1 binding activity, and AP-1 heterodimer formation. This study thus describes multiple levels of regulation of the AP-1 system after precipi-

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ABBREVIATIONS: AP-1, activator protein-1; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; bp, base pair(s); 2-DG, 2-deoxyglucose; CRE, cAMP-responsive element; SP-1, SV40 promoter factor-1; CTF/NF-1, cellular transcription factor/nuclear factor-1.

tated withdrawal from morphine and suggests a possible genomic involvement regarding neural plasticity associated with morphine dependence.

Materials and Methods

Animals and Morphine Administration Paradigms

Adult (90-day-old) male Sprague-Dawley rats were kept under a 12-hr light/12-hr dark cycle and given food and water *ad libitum*. Morphine was chronically administered by subcutaneous implantation of morphine pellets (75 mg of base; National Institute on Drug Abuse) (a gift from Dr. John Williams, Vollum Institute) under light halothane anesthesia. Precipitated withdrawal was examined with two morphine administration paradigms. Schedule 1 treatment consisted of implantation of one morphine pellet every day for 7 days, followed by an intraperitoneal injection of naloxone (Sigma Chemical Co., St. Louis, MO) 24 hr later. Schedule 2 morphine treatment consisted of implantation of one, two, and three morphine pellets on days 1, 4, and 7, respectively, followed by an intraperitoneal injection of naloxone on day 10. Control groups received placebo pellets, saline injections, or both. At 1 and 6 hr after the administration of naloxone, the whole brain was removed and rinsed in ice-cold phosphate-buffered saline for 1 min before dissection. The cortex (cingulate, motor, parietal, temporal, and occipital regions), cerebellum, hippocampus, striatum, hypothalamus, midbrain/thalamus, and hindbrain were dissected and immediately stored on dry ice. All tissue samples were stored at −70°.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from the aforementioned brain regions, and RNA concentrations were determined spectrophotometrically. For Northern blot analysis, total RNA (7–10 μg) was separated by electrophoresis on 6% formaldehyde/1.2% agarose gels. The RNA was transferred to Magna NT nylon membranes (Micron Separations, Westboro, MA) by capillary action with 5× standard saline citrate (750 mM Sodium Chloride and 75 mM Sodium Citrate, pH 7.0), followed by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA). The membranes were then briefly dipped in 0.3 M sodium acetate, 0.02% methylene blue, to stain the transferred RNA. The 28 S and 18 S rRNAs were readily apparent and served as a means to normalize for the amount of RNA contained within each sample. After removal of stain by boiling in water for 10 min, the membranes were prehybridized for 3–24 hr at 60° in hybridization buffer [5% sodium dodecyl sulfate, 400 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mg/ml bovine serum albumin (fraction V), 50% formamide]. The prehybridization buffer was discarded and replaced with fresh hybridization buffer including a specific cRNA hybridization probe. Hybridization proceeded for 16–24 hr at 60°. Membranes were subsequently washed with 1% sodium dodecyl sulfate, 0.05× standard saline citrate, 1 mM EDTA, at 70–75° for 1–4 hr. After washing, membranes were exposed multiple times to Kodak XAR-5 film to obtain a range of hybridization signal intensities for semiquantitative densitometric analysis. For each RNA sample, autoradiographic signals within the linear range of film sensitivity were digitized using a tabletop laser scanner (model MSF300ZS; X-Ray Scanner Corp.) and Adobe Photoshop XSF software. Relative intensities were quantified using Image software. Immediate early gene mRNA signal intensities were standardized against cyclophilin mRNA (detected by Northern blot analysis using radiolabeled cRNA as a hybridization probe) (18) or 28 S and 18 S rRNAs (detected by methylene blue staining) for each sample.

In Situ Hybridization

For *in situ* hybridization analysis, a slightly modified version of the protocol of Simmons *et al.* (19) was used. The main variation was storage of cut, free-floating sections in cryoprotectant (50% 0.05 M sodium phosphate buffer, pH 7.3/30% ethylene glycol/20% glycerol) at −20° for 2–6 weeks. Sections were then mounted onto 0.1% gelatin- and poly-L-lysine-coated slides, washed three times in 0.05 M sodium phosphate buffer, pH 7.3, vacuum dried, and stored desiccated at −70° until hybridization. Serial sections (30-μm thick) were cut on a sliding microtome. Whole-brain series were collected from the olfactory bulb to the cervical spinal cord, and each section in a series was approximately 270 μm from the adjacent section. Hybridization with c-*fos* cRNA probes was performed at 67°, with the most stringent posthybridization wash step being performed at 78°. The cRNA probes were synthesized from the c-*fos* cDNA construct described below. Hybridization with a sense c-*fos* RNA probe served as a control for c-*fos* mRNA signal specificity.

Gel Shift Assays

Whole-cell protein extracts. Two groups of five adult male Sprague-Dawley rats were each given placebo pellets, followed by saline injection, or schedule 1 morphine treatments plus naloxone (100 mg/kg) and were sacrificed 2 hr later. The brain was dissected and homogenized (Dounce homogenizer) in buffer A (0.25 M sucrose, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 150 μM spermine, 500 μM spermidine, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 0.1 mM *p*-aminobenzamide) on ice. Extracts were centrifuged at 2000 × *g* for 10 min. The resulting pellet was resuspended in buffer B (0.5 M HEPES, pH 7.9, 0.75 mM $MgCl_2$, 0.5 mM EDTA, 0.5 M KCl, with protease inhibitors) by gentle mixing for 30 min, followed by centrifugation at 14,000 × *g* for 30 min. The resulting supernatant was dialyzed for 2 hr against 1 liter of 10 mM Tris·HCl, 7.9, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, with protease inhibitors, with a change of dialysis buffer after 1 hr. All of the aforementioned manipulations were performed at 4°. Samples were divided into aliquots, flash frozen in a dry ice/95% ethanol bath, and stored at −70°. Protein concentrations were determined with the Bio-Rad protein assay.

Gel retention analysis. Various amounts of whole-cell protein extracts were mixed with binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 1% glycerol, with 0.5 mM DTT added fresh each time), 1 μg of poly(dI·dC), and 1 × 10^4 cpm of a double-stranded ^{32}P-labeled AP-1 oligonucleotide (CGCTTGATGAGTCAGCCGGAA) (Bold residues represent consensus AP-1 recognition site; Promega, Madison, WI), in a final volume of 20 μl. (Competing nonradiolabeled oligonucleotides containing AP-1, CRE, CTF/NF-1, and SP-1 consensus sequence elements were also purchased from Promega.) For supershift assays, the mixture also contained rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) recognizing specific Fos (c-Fos and Fos-B) and Jun (c-Jun, Jun-B, and Jun-D) proteins. The reactions mixtures were incubated at 37° for 15 min, mixed with gel loading buffer, and subjected to electrophoresis on a 4% acrylamide gel. Gels were then dried and exposed to Kodak XAR-5 film for multiple autoradiographic exposures.

Antiserum specificity. Antiserum specificity was analyzed by various means. The antisera employed in these studies were raised against synthetic 13–17-residue peptides that represent unique sequence elements of the known Fos and Jun proteins. Thus, antisera directed against any specified Fos or Jun protein epitope would not be expected to cross-react with other members of the family. One characterization study used epitope-specific and nonspecific peptides in AP-1 DNA binding reactions (data not shown). For every antiserum utilized, only the epitope-specific peptide was able to successfully compete for the formation of a supershifted complex, whereas nonrelated epitope peptides had no effect. Other immunochemical forms of analysis further support the notion of specificity.[1] Nuclear and cytoplasmic extracts have been prepared from a wide variety of mammalian cell lines (e.g., KNRK, HeLa, A431, and NIH/3T3 cells) and subjected to both immunoprecipitation and Western blot analyses. Results from both types of analysis further confirm that antiserum directed against a given Fos or Jun protein is unable to recognize other members of the family. However, this analysis does not rule out the possibility that other

[1] Seth Horwitt, Santa Cruz Biotechnology, personal communication.

cellular proteins unrelated to Fos or Jun proteins are capable of cross-reactivity.

AP-1 oligonucleotide radiolabeling. Double-stranded consensus AP-1 oligonucleotide was end-labeled in 50 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, with T4 polynucleotide kinase (BRL, Bethesda, MD) and [γ-^{32}P]ATP (6000 Ci/mmol).

Plasmid Constructs and Riboprobe Synthesis

Analysis of relative *fos* and *jun* mRNA levels was carried out with cRNA probes after subcloning of specific DNA fragments into the plasmid pGEM-3Z (Promega). Full length rat cDNA clones for c-*fos* and c-*jun* were a generous gift from Dr. Tom Curran (Roche Institute, Nutley, NJ). A 1255-bp fragment (bases 1–1255, *Eco*RI/*Sac*I) of the c-*fos* clone and a 817-bp fragment (bases 1–817, *Eco*RI/*Pst*I) of the c-*jun* clone were subcloned. All other clones were of mouse origin and were obtained from the American Type Culture Collection (Rockville, MD). For *jun*-D, a 899-bp *Sph*I fragment (bases 323–1222) was subcloned. Polymerase chain reaction-amplified fragments (with *Hind*III/*Xba*I ends) of *fos*-B (bases 73–1206) and *jun*-B (bases 284–1390) were used for subcloning. A 650-bp fragment (bases 35–685, *Pst*I/*Hinc*II) of rat cyclophilin cDNA was subcloned. Radiolabeled ([α-^{32}P]UTP) cRNA probes were synthesized *in vitro* using SP6, T3, or T7 RNA polymerase. Specific activities of cRNA probes were routinely >3 × 10^9 cpm/μg of plasmid.

Statistical Analysis

Normalized values are represented as means ± standard errors, unless otherwise stated. Statistical analyses were determined with a one-way analysis of variance, using the Fischer protected least-significant difference test, as calculated with Statview II software.

Results

Expression of *fos* and *jun* genes after naloxone-precipitated morphine withdrawal. Naloxone-precipitated morphine withdrawal elicited increases in rat brain c-*fos* mRNA levels, as determined by Northern blot analysis. Fig. 1A documents such increases in c-*fos* mRNA levels from individual animals 1 hr after naloxone-precipitated withdrawal. These animals received one morphine pellet daily for 7 days, followed by treatment with various doses of naloxone 24 hr after implantation of the last pellet (schedule 1). Apparent variability in the degree of c-*fos* mRNA induction by naloxone (particularly at the 1 mg/kg dose) is due to differential responses between individual animals, because equivalent amounts of total RNA were present in the samples shown. The hypothalamus and cerebellum showed the greatest relative increase in steady state c-*fos* mRNA levels, followed by the hippocampus, midbrain/thalamus, striatum, and cortex (Fig. 1B). Within the hindbrain, the only observed change (2-fold) occurred within the morphine plus 100 mg/kg naloxone group (data not shown). Control groups showed that stress (placebo plus saline), naloxone itself (placebo plus naloxone), and chronic morphine treatment (morphine plus saline) had little effect on c-*fos* mRNA levels. Thus, in this study it was documented that brain region-specific changes in c-*fos* mRNA levels associated with precipitated withdrawal are proportional to the naloxone dose given to rats chronically treated with morphine.

To further assess the nature of c-*fos* mRNA induction after precipitated morphine withdrawal, a more complete naloxone dose-response analysis was performed, with cortex representing the specific brain area examined. With morphine implantation schedule 2 (one, two, and three pellets implanted on days 1, 4, and 7, respectively, and withdrawal precipitated on day 10), the

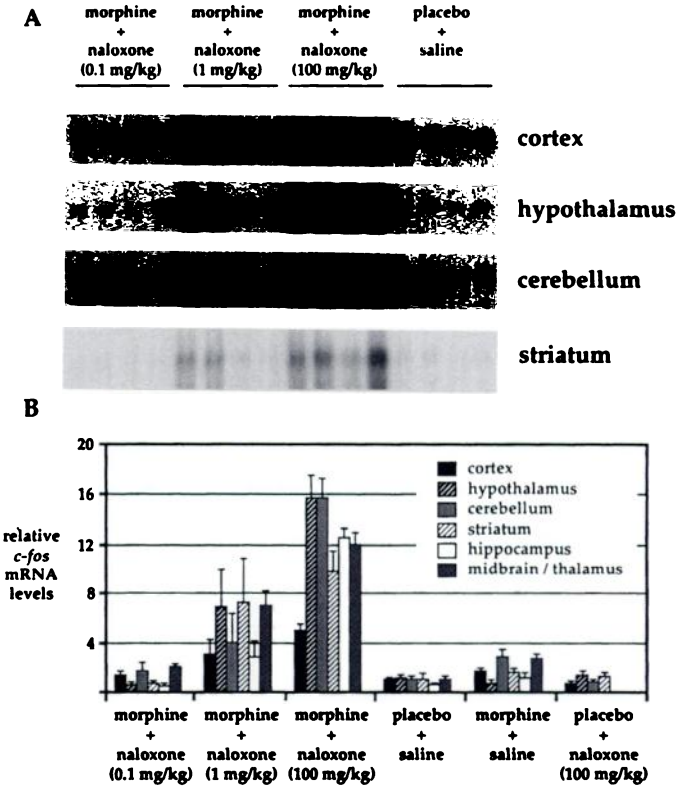

Fig. 1. Naloxone-precipitated morphine withdrawal regulates c-*fos* mRNA levels throughout the rat brain. Rats chronically treated with morphine implantation schedule 1 (one pellet daily for 7 days and intraperitoneal naloxone injections on day 8) were sacrificed 1 hr after precipitation of withdrawal, and RNA was isolated and processed for Northern blot analysis. Control animals received placebo pellets and saline on day 8 (*placebo + saline*), morphine pellets and saline on day 8 (*morphine + saline*), or placebo pellets and 100 mg/kg naloxone on day 8 [*placebo + naloxone (100 mg/kg)*]. A, Autoradiograms of c-*fos* mRNA signals from individual animals (n = 4). B, Results from semiquantitative densitometric analysis, representing the means ± standard errors of c-*fos* mRNA levels, relative to the placebo plus saline group (n = 4). The relative levels of c-*fos* mRNA in the hippocampus and midbrain/thalamus for the placebo plus naloxone (100 mg/kg) group were not measured. Hindbrain results are not shown because c-*fos* mRNA regulation by precipitated withdrawal was minor by comparison.

timing and dose of morphine administration were varied. A more thorough c-*fos* mRNA dose-response curve during withdrawal was determined by administering naloxone over a 10,000-fold range (Fig. 2). The two morphine implantation schedules were comparable in their ability to induce physical signs of withdrawal (see below) and in their ability to affect c-*fos* mRNA levels after morphine withdrawal. There is little difference in the shape of the curves in Fig. 2, although there was a slightly greater level of c-*fos* mRNA induction with schedule 1. Although only cortical responses are illustrated, c-*fos* mRNA induction within other brain regions after precipitated withdrawal were similar between the two morphine pellet implantation schedules (data not shown). Therefore, schedule 2 results suggest that the c-*fos* transcriptional responses within the brain after precipitated morphine withdrawal exhibit classical dose-dependent characteristics.

A second member of the *fos* gene family, *fos*-B, was also examined and exhibited little, if any, relative change in brain mRNA levels after naloxone-precipitated withdrawal. Northern blot analysis was unable to detect any brain region-specific

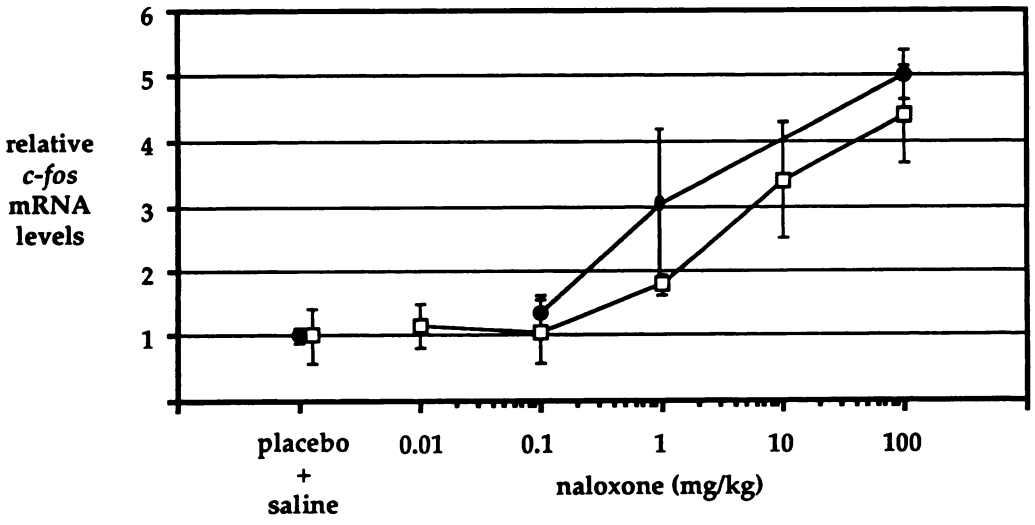

Fig. 2. Induction of c-*fos* mRNA levels by precipitated morphine withdrawal varies as a function of naloxone dose. The effect of naloxone dose on c-*fos* mRNA levels within the cortex of animals treated with morphine implantation schedule 1 (●) was compared with that in animals treated with a second chronic morphine administration protocol (schedule 2; one, two, and three morphine pellets administered on days 1, 4, and 7, respectively, followed by intraperitoneally administered naloxone on day 10) (□). Each *point* represents the mean ± standard error of c-*fos* mRNA levels, relative to the placebo plus saline group ($n = 4$).

changes of >2-fold. In the cortex and cerebellum, however, there were approximately 2–3-fold and 3–5-fold increases in *fos*-B mRNA levels, respectively, after administration of the highest dose of naloxone with either morphine implantation schedule.

Of the three *jun* family genes (c-*jun*, *jun*-B, and *jun*-D) examined, naloxone-precipitated withdrawal affected *jun*-B mRNA levels to a much greater extent, compared with those of c-*jun* and *jun*-D. Brain region-specific changes in *jun*-B mRNA levels after precipitated withdrawal were restricted to the cortex (Fig. 3A), hippocampus, and striatum (Fig. 3B), as shown for animals treated with morphine implantation schedule 1. In Fig.

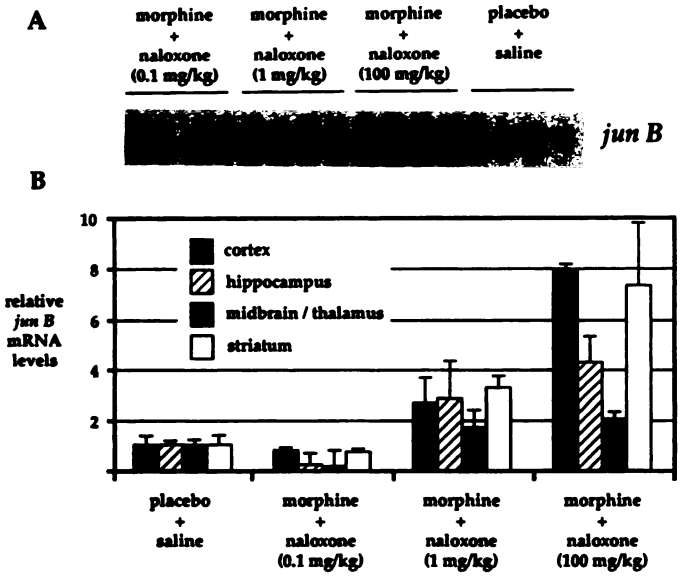

Fig. 3. Naloxone-precipitated morphine withdrawal regulates *jun*-B mRNA levels in limited brain areas. Rats chronically treated with morphine implantation schedule 1 (one pellet daily for 7 days and intraperitoneal naloxone injections on day 8) were sacrificed 1 hr after precipitation of withdrawal, and RNA was isolated and processed for Northern blot analysis. Control animals received placebo pellets and saline on day 8 (*placebo + saline*). A, Autoradiogram of *jun*-B mRNA signals from the cortex of individual animals. B, Results from semiquantitative densitometric analysis, representing the means ± standard errors of *jun*-B mRNA levels, relative to the placebo plus saline group ($n = 4$). Neither basal nor stimulated signals for *jun*-B mRNA were detected in the cerebellum. The hindbrain exhibited no *jun*-B regulation after precipitated withdrawal.

3A, similarly to results observed for c-*fos* mRNA levels, apparent variability in the degree of *jun*-B mRNA induction by naloxone (particularly at the 1 mg/kg dose) is due to differential responses between individual animals; equivalent amounts of total RNA were present in the samples shown. The levels of *jun*-B mRNA were unaltered in the midbrain/thalamus by precipitated withdrawal and were undetectable in the hypothalamus and cerebellum. Thus, *jun*-B mRNA levels exhibit brain region-selective regulation after precipitated withdrawal, with the degree of change being directly proportional to the dose of naloxone administered. As with c-*fos* mRNA changes, *jun*-B mRNA increases were not due to injection/implantation stress (saline plus placebo) or the independent administration of either morphine or naloxone (data not shown). There was little change in steady state levels of c-*jun* mRNA after precipitated withdrawal, with observed changes occurring only within the cortex (Fig. 4). A 3-fold increase in c-*jun* mRNA levels was seen in the cortex of animals treated with morphine pellets (schedule 1) followed by administration of the highest naloxone dose (100 mg/kg). No changes in *jun*-D mRNA levels were observed in the brain regions tested after any morphine withdrawal paradigm. Therefore, precipitated morphine withdrawal further appears to selectively activate a subset of *jun* family genes in a brain region-specific manner.

Morphine dependence after establishment of the two morphine pellet implantation schedules was also evaluated, by assessing physical symptoms of withdrawal. We subjectively determined the presence and relative intensity of withdrawal symptoms, such as wet-dog shakes, lacrimation, salivation, oral stereotypies, piloerection, vocalization to touch, and diarrhea, at 1 and 6 hr after naloxone administration. At the lower naloxone doses (0.01 and 0.1 mg/kg), a few wet-dog shakes (1–2 within the first 1 hr) were seen in schedule 1-treated animals, but no apparent withdrawal signs were noted with schedule 2-treated animals. At 1 mg/kg naloxone, both groups exhibited wet-dog shakes and lacrimation. Schedule 1-treated animals also displayed oral stereotypies, whereas schedule 2-treated animals had diarrhea and vocalizations to touch. In schedule 2-treated animals, 10 mg/kg naloxone produced salivation, diarrhea, and vocalization to touch but with greater intensity than at 1 mg/kg naloxone. At the highest naloxone dose (100 mg/kg), both schedule 1- and 2-treated groups exhibited salivation,

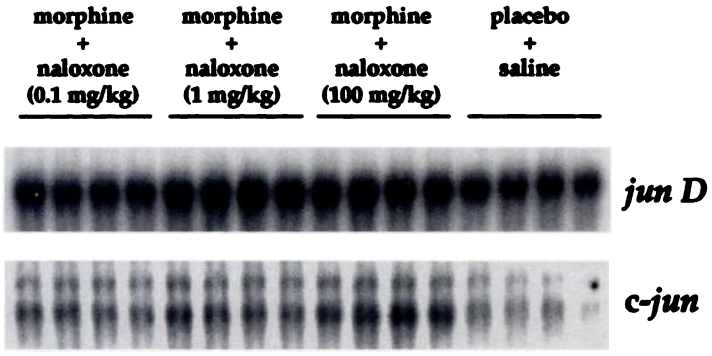

Fig. 4. Naloxone-precipitated morphine withdrawal regulates c-*jun* but not *jun*-D mRNA levels in the cortex. Rats chronically treated with morphine implantation schedule 1 (one pellet daily for 7 days and intraperitoneal naloxone injections on day 8) were sacrificed 1 hr after precipitation of withdrawal, and RNA was isolated and processed for Northern blot analysis. Control animals received placebo pellets and saline on day 8 (*placebo + saline*). The autoradiogram shows *jun*-B and *jun*-D mRNA signals from the cortex of individual animals.

lacrimation, wet-dog shakes, and diarrhea with greater intensity than at the lower naloxone doses used. These physical signs of withdrawal are consistent with those reported by Bläsig and coworkers and by several others at equivalent doses of morphine and naloxone (20–24). Thus, the changes in *fos* and *jun* mRNA levels not only were naloxone dose dependent but also were proportional to the intensity of precipitated withdrawal.

***In situ* hybridization analysis of c-*fos* mRNA expression.** To obtain a higher level of neuroanatomical resolution, *in situ* hybridization analysis of c-*fos* mRNA expression was performed. c-*fos* mRNA was chosen for analysis because this transcript exhibited the most dynamic response to precipitated withdrawal. As determined by *in situ* hybridization, precipitated withdrawal (schedule 1 plus 100 mg/kg naloxone, $n = 6$) produced intense c-*fos* mRNA signals within numerous brain regions (Fig. 5; Table 1). Within the hypothalamus, the most dense hybridization signals were observed in the paraventricular nucleus, suprachiasmatic nucleus, supraoptic nucleus, posterior hypothalamus, and lateral hypothalamic area. Most cortical areas also exhibited c-*fos* mRNA signals, as did the granule cell layer of the cerebellum. A number of limbic structures, such as the amygdaloid complex, hippocampal formation, septum, habenula, nucleus accumbens, ventral tegmental area, substantia nigra, and interpenduncular complex, exhibited intense to moderate c-*fos* mRNA signals. Of the mesencephalic structures, only the medial half of the ventral tegmental area, the medial third of the substantia nigra pars reticulata, and scattered neurons within the pars compacta showed c-*fos* mRNA signals. A few autonomic brains regions, such as the locus ceruleus, the area postrema, and the nucleus of the solitary tract, also revealed intense c-*fos* mRNA signals. Very few sites within the thalamus exhibited c-*fos* mRNA signals after withdrawal, with the exception of the habenula and geniculate. In less severely withdrawn animals (schedule 1 and 1 mg/kg naloxone, $n = 3$), c-*fos* mRNA signals above control values were limited to the cortex and hippocampal formation (data not shown). Control animals (placebo plus saline, $n = 4$) exhibited moderate c-*fos* mRNA signals in the piriform cortex, neocortex, and pontine gray.

AP-1 DNA binding analysis after naloxone-precipitated morphine withdrawal. The genomic target of homo- and heterodimers composed of Fos and Jun transcription factors is the AP-1 DNA recognition site (TGAGTCA). Gel shift assays using an AP-1 consensus oligonucleotide and whole-cell protein extracts from various brain regions were performed to serve as a semifunctional assessment of AP-1 DNA-binding activity present in those brain structures after precipitated

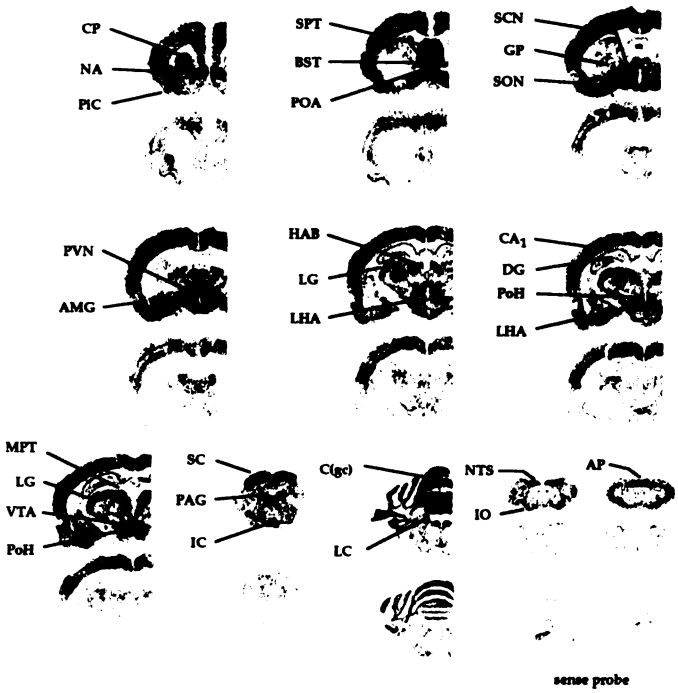

Fig. 5. *In situ* hybridization analysis of c-*fos* mRNA expression after naloxone-precipitated morphine withdrawal provides higher level of neuroanatomical resolution. Rats chronically treated with morphine implantation schedule 1 (one pellet daily for 7 days and intraperitoneal naloxone injections on day 8) were sacrificed 1 hr after naloxone administration (100 mg/kg) and processed for *in situ* hybridization (see Materials and Methods) ($n = 6$). One section through the cerebellum and hindbrain is shown after hybridization with a ^{35}S-labeled sense c-*fos* cRNA probe (*lower right*). Each grouping of two representative autoradiograms is from 30-μm serial sections of an animal undergoing withdrawal (*top*), as described above, and a control animal (placebo plus saline) (*bottom*). Autoradiographic images were scanned with a 35-mm slide scanner and digitally reproduced. Brain structures exhibiting enhanced c-*fos* mRNA signals after withdrawal are shown (*lines*). *AMG*, amygdala; *AP*, area postrema; *BST*, Bed nucleus stria terminalis; *C(gc)*, cerebellar granule cell layer; *CA*$_1$, Ammon's horn, field 1; *CP*, caudate putamen; *DG*, dentate gyrus; *GP*, globus pallidus; *HAB*, habenula; *IC*, interpenduncular complex; *IO*, inferior olive; *LC*, locus ceruleus; *LG*, lateral geniculate; *LHA*, lateral hypothalamic area; *MPT*, medial pretectal area; *NA*, nucleus accumbens; *NTS*, nucleus tractus solitarus; *PAG*, periaqueductal gray; *PIC*, piriform cortex; *POA*, preoptic area; *PoH*, posterior hypothalamic area; *PVN*, paraventricular nucleus of the hypothalamus; *SC*, superior colliculus; *SCN*, suprachiasmatic nucleus; *SON*, supraoptic nucleus; *SPT*, septum; *VTA*, ventral tegmental area.

TABLE 1

Relative intensity of c-*fos* mRNA signals in rat brain 1 hr after naloxone-precipitated morphine withdrawal

Data represent relative increases in c-*fos* mRNA signal density in naloxone-treated animals (schedule 1 + 100 mg/kg naloxone), compared with controls (placebo + saline).

Brain region	mRNA signal intensity[a]
Telencephalon	
Cortex	
Frontal	++
Cingulate	++
Piriform	+++
Parietal	+
Entorhinal	+
Temporal	+
Occipital	++
Amygdala	
Central nucleus	++++
Medial nucleus	+++
Lateral nucleus	0
Basolateral nucleus	0
Cortical nucleus	++
Hippocampal formation	
CA1, CA3	+++
CA2	++
Dentate gyrus, granule cell layer	+++
Endopiriform nucleus	+
Olfactory tubercle	+
Claustrum	0
Nucleus accumbens	++
Caudate putamen (dorsolateral)	++
Globis pallidus	+
Septum	
Medial	++++
Lateral	++++
Subfornical organ	++
Stria terminalis	0
Bed nucleus stria terminalis	++
Preoptic area	
Medial	+++
Lateral	+++
Diencephalon	
Hypothalamus	
Anterior hypothalamic area	0
Supraoptic nucleus	++++
Suprachiasmatic nucleus	++++
Paraventricular nucleus	++++
Periventricular nucleus	+
Arcuate nucleus	+
Median eminence	0
Ventromedial nucleus	0
Dorsomedial nucleus	+++
Lateral hypothalamic area	++++
Mamillary nucleus	0
Posterior hypothalamus	+++
Supramamillary nucleus	0
Thalamus	
Stria medullaris	+
Mediodorsal nucleus	++
Central medial nucleus	++
Geniculate	
Medial	0
Lateral	++++
Medial pretectal area	+++
Habenula	
Medial	+
Lateral	++++
Zona incerta	+
Mesencephalon	
Interpenducular complex	+++
Substantia nigra	
Pars compacta	+
Pars reticulata	0
Ventral tegmentum	++
Periaqueductal grey	
Dorsal	+
Ventral	+++
Superior colliculus	++++
Inferior colliculus	0
Rhombencephalon	
Locus ceruleus	++++
Area postrema	++++
Inferior olive	+++
Nucleus solitary tract	+++
Cerebellum granule cell layer	++++

[a] ++++, very dense; +++, dense; ++, moderately dense; +, minimally dense; 0, not detectable.

morphine withdrawal. The use of antibodies directed against Fos and Jun transcription factors allowed the further identification of those Fos and Jun family members comprising the observed AP-1 DNA/protein complexes.

Brain region-specific increases in AP-1 binding were observed 2 hr after precipitated withdrawal (morphine pellet implantation schedule 1 plus 100 mg/kg naloxone, $n = 5$). Although AP-1 binding activity was detectable in all brain areas tested, relatively significant increases were localized to the cortex, cerebellum, hippocampus, and striatum (Fig. 6; Table 2), No noticeable differences were observed, though, in the midbrain/thalamus and hindbrain, compared with controls (placebo plus saline). Hypothalamic extracts were not studied because of cellular heterogeneity and low tissue abundance. Oligonucleotide competition studies with unlabeled AP-1-specific and nonspecific oligonucleotides confirmed the AP-1 sequence-specific nature of complex formation after gel retention analysis (Fig. 7). Non-AP-1-related sequence elements (CTF/NF-1 and SP-1) were unable to compete for complex formation at all concentrations tested. A CRE-containing oligonucleotide served as a competing oligonucleotide, as expected, due to the significant similarity between concensus AP-1 (TGAGTCA) and CRE (TGACGTCA) sequence recognition elements.

A series of antibody supershift studies were next performed to begin to identify the specific Fos and Jun protein factors that constitute the retained AP-1 complexes formed from brain region protein extracts. This type of analysis makes use of serum IgG antibodies that are directed against synthetic peptides representing unique sequences within the various characterized Fos and Jun proteins (see Materials and Methods for a more detailed description of antiserum characterization and specificity). The antiserum is added to the binding reaction before gel electrophoresis and, if a peptide epitope to which the antiserum is directed is present within the retained AP-1 DNA complex, then the antibody binds to the complex and a supershifted band results after autoradiography. A corresponding reduction in the relative intensity of the radiolabeled AP-1 DNA complex may also occur (even without the appearance of a supershifted band), thus serving as an additional indication of the presence of a specific antigen within the AP-1 complex. Presumably, the peptide epitope represents a unique segment of the targeted Fos or Jun protein, although cross-reactivity with a nonrelated AP-1 DNA-binding protein cannot be formally excluded. Additionally, although the presence of a supershifted band represents a qualitative evaluation of a specific Fos or Jun protein, the lack of appearance of a supershifted product does not necessarily imply the absence of that protein; epitopes could be masked by post-translational modification events or through association with other cellular proteins.

Supershifted bands presumably corresponding to Fos- and

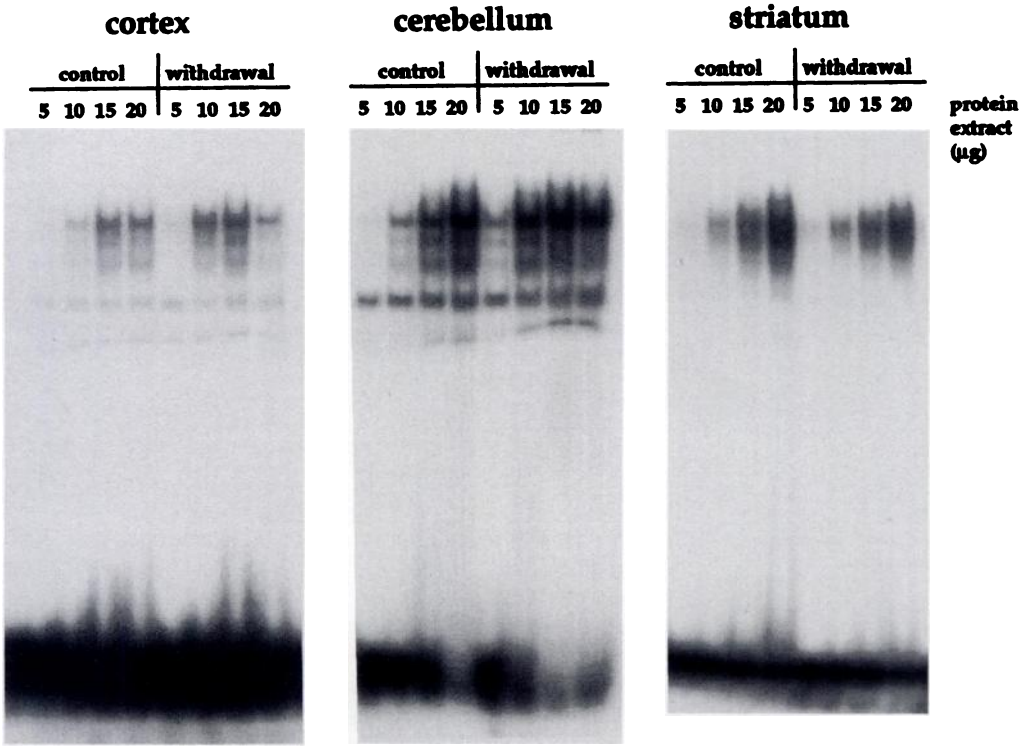

Fig. 6. Precipitated morphine withdrawal increases AP-1 DNA-binding activity in the cortex, cerebellum, and striatum. Gel retention analysis of AP-1 DNA/protein complexes was performed with a ^{32}P-end-labeled consensus AP-1 oligonucleotide and increasing protein concentrations (5, 10, 15, and 20 μg) of whole-cell extracts from tissues isolated from animals 2 hr after precipitated withdrawal (schedule 1 plus 100 mg/kg naloxone) (*withdrawal*) or from control animals (placebo plus saline) (*control*). AP-1 DNA/protein complexes are visualized at the *top of the autoradiograph*, whereas the more quickly migrating unbound oligonucleotide is at the *bottom*.

TABLE 2

Approximate fold increases in AP-1 DNA/protein-binding activity within individual brain regions after naloxone-precipitated withdrawal

Data represent relative increases in AP-1 DNA-binding activity from naloxone-treated animals (scheule 1 plus 100 mg/kg naloxone) (n = 5), compared with controls (placebo plus saline) (n = 5). Values were determined by semiquantitative densitometric analysis of autoradiographic signals similar to those presented in Fig. 6.

	AP-1 binding activity
	fold increase
Cortex	7.1
Cerebellum	4.0
Hippocampus	2.3
Striatum	1.3
Midbrain/thalamus	NC[a]
Hindbrain	NC

[a] NC, no change.

Jun-containing AP-1/DNA complexes exhibited a selective brain region distribution and regulation by precipitated withdrawal. A representative immediate early gene supershift analysis is shown in Fig. 8, where protein extracts from the cortex of both control and naloxone-treated animals exhibit supershifted bands for all of the Fos and Jun transcription factors examined. Identical analyses were performed with protein extracts from cerebellum, hippocampus, striatum, midbrain/thalamus, and hindbrain after isolation from both control and naloxone-treated animals (data not shown). A qualitative analysis of the results can be summarized as follows. Two hours after precipitated withdrawal, c-Fos-containing AP-1 complexes were significantly increased in the cerebellum, cortex, hippocampus, and striatum. Detectable levels of Fos-B-containing AP-1 complexes from control extracts were limited to the cortex, striatum, and hippocampus, with morphine withdrawal promoting an increase in such complexes in the cortex and hippocampus. Of the three Jun factors examined, Jun-B-containing AP-1 complexes exhibited a profile of appearance similar to that of Fos-B-containing AP-1 complexes, although precipitated withdrawal caused an increase in Jun-B-containing AP-1 complexes in the cortex, striatum, and hippocampus. In contrast, Jun-D-containing AP-1 complexes were present in all brain areas studied, with relative levels being positively regulated by precipitated withdrawal in the cortex, cerebellum, hippocampus, and midbrain/thalamus. Lastly, c-Jun-containing AP-1 complexes were barely detectable in all brain regions examined, with a minimal increase being observed in the cerebellum and cortex after morphine withdrawal. These data thus document a brain region-specific distribution of the individual Fos and Jun proteins that comprise the AP-1 DNA complexes, with selective regulation of the relative levels of the individual transcription factors being observed after precipitated morphine withdrawal.

An additional level of analysis of AP-1 DNA/protein complex composition was performed through the combined use of Fos/Jun antibodies in supershift experiments. This analysis was limited to the cortex, cerebellum, and striatum, because of their robust transcriptional responses, relative cellular homogeneity, and putative function in morphine dependence. A subset of presumed Fos/Jun heterodimers appear to be differentially distributed in control extracts, with the apparent levels of some of these complexes exhibiting positive regulation after naloxone-precipitated withdrawal. A representative combination supershift analysis is shown in Fig. 9, where protein extracts from the cortex of both control and naloxone-treated animals exhibit supershifted products indicative of the formation of AP-1 heterodimers. Similar analyses were performed with protein extracts from cerebellum and striatum after isolation from both control and naloxone-treated animals (data not shown). A qualitative analysis of the results can be summarized as follows. The cortex exhibited the most varied array of c-Fos/

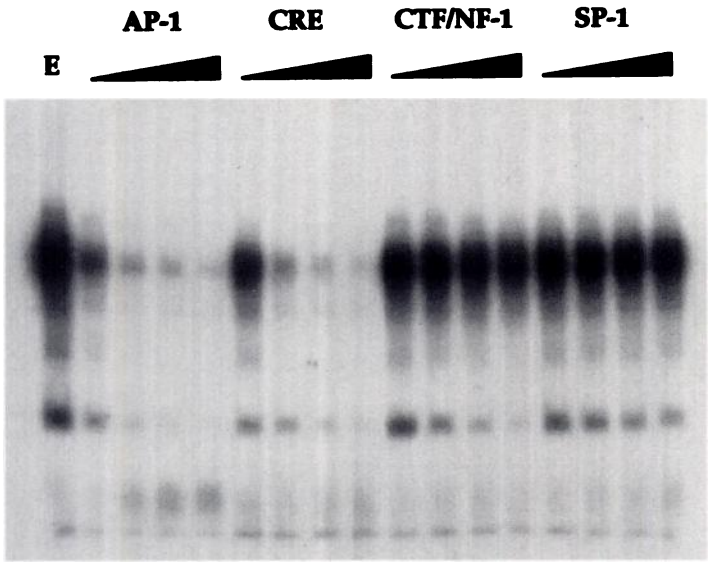

Fig. 7. AP-1 sequence-specific DNA/protein complexes are present in whole-cell extracts, as determined by oligonucleotide competition analysis. Protein extracts (10 μg) prepared from the cerebellum 2 hr after precipitated withdrawal (schedule 1 plus 100 mg/kg naloxone) were preincubated with a 10-, 50-, 100-, or 250-fold molar excess of nonradiolabeled AP-1, CRE, CTF/NF-1, or SP-1 oligonucleotide before incubation with the ^{32}P-end-labeled consensus AP-1 oligonucleotide. The free unbound probe was electrophoresed out of the gel. *E*, probe plus extract alone.

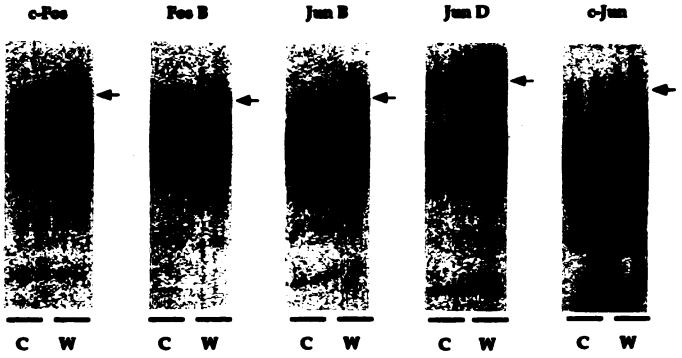

Fig. 8. Precipitated morphine withdrawal differentially regulates the apparent relative levels of cortical Fos and Jun proteins capable of forming AP-1 DNA/protein complexes. Polyclonal antisera against peptide epitopes of c-Fos, Fos-B, Jun-B, Jun-D, and c-Jun were added to preformed radiolabeled AP-1 DNA/protein complexes before electrophoresis. Cortical extracts were prepared from animals 2 hr after precipitated withdrawal (schedule 1 plus 100 mg/kg naloxone) (n = 5) (*W*) or from control animals (placebo plus saline) (n = 5) (*C*). The presence of Fos and Jun proteins within AP-1 DNA/protein complexes results in a more slowly migrating (or supershifted) complex (*arrows*). Presented results represent two different amounts (10 and 15 μg) of cortical extracts from both control and naloxone-treated animals.

Jun (Jun-B and Jun-D) and Fos-B/Jun (c-Jun, Jun-B, and Jun-D) heterodimers of the three brain areas studied. Detectable striatal AP-1 heterodimer formation was observed for c-Fos/Jun-B, Fos-B/c-Jun, Fos-B/Jun-B, and Fos B/Jun-D, whereas detectable cerebellar AP-1 heterodimers were limited to c-Fos/Jun-B and c-Fos/Jun-D. The most dramatic induction of heterodimer formation within these brain regions after naloxone-precipitated withdrawal was for c-Fos/Jun-B in the cortex and striatum and c-Fos/Jun-D in the cerebellum. These results thus suggest the differential distribution and morphine withdrawal-sensitive regulation of AP-1 heterodimer levels throughout the brain.

Discussion

This study finds that neural plasticity associated with morphine dependence involves brain region-specific regulation of the AP-1 system. At the level of transcription, naloxone-precipitated morphine withdrawal induced expression of a subset of *fos* and *jun* genes (e.g., c-*fos* and *jun*-B), with the relative degree of induction varying as a function of the severity of physical dependence. The most dynamic changes in gene expression after withdrawal were seen with c-*fos* mRNA, followed by *jun*-B mRNA, which showed a more restricted pattern of regulation. These responses suggest a role for c-*fos* and *jun*-B as transcriptional indicators of the severity of morphine withdrawal in the brain. A more limited pattern of regulation was evident for c-*jun* and *fos*-B mRNA levels after withdrawal, whereas *jun*-D mRNA levels remained unaffected. Therefore, because only a subset of *fos* and *jun* genes are activated during precipitated withdrawal and response levels correlate with the severity of withdrawal, these transcriptional responses appear to represent more than just cellular stress responses. Furthermore, these transcriptional changes were localized to specific brain structures relevant to symptoms of physical dependence.

Another means of ascertaining the specificity of transcriptional changes was to examine the specific cellular localization of c-*fos* mRNA expression within the brain by *in situ* hybridization analysis. One could predict that enhanced c-*fos* mRNA expression would occur within neural circuits thought to mediate some of the behaviors associated with morphine withdrawal (25, 26). Indeed, precipitated withdrawal induced c-*fos* mRNA levels within neuronal circuits mediating osmolality, feeding, reward and motivation, arousal, and cognition, i.e., behaviors affected by morphine withdrawal (2, 3). The suprachiasmatic nucleus, which participates in regulating circadian rhythms (27), and the locus ceruleus, whose activity modulates arousal (28), exhibited dramatic increases in c-*fos* mRNA signals, compared with control groups. Enhanced c-*fos* mRNA induction was also observed within the lateral hypothalamic area, which subserves feeding behaviors, and the paraventricular nucleus of the hypothalamus, which assists with the regulation of osmolality (29). The hippocampus and prefrontal cortex, which participate in acquisition, processing, and storage of memory (30), also exhibited robust c-*fos* mRNA expression. The midbrain ventral tegmentum and the nucleus accumbens, which subserve the rewarding and motivational aspects of drugs of abuse (25, 26, 31), exhibited moderate increases in c-*fos*

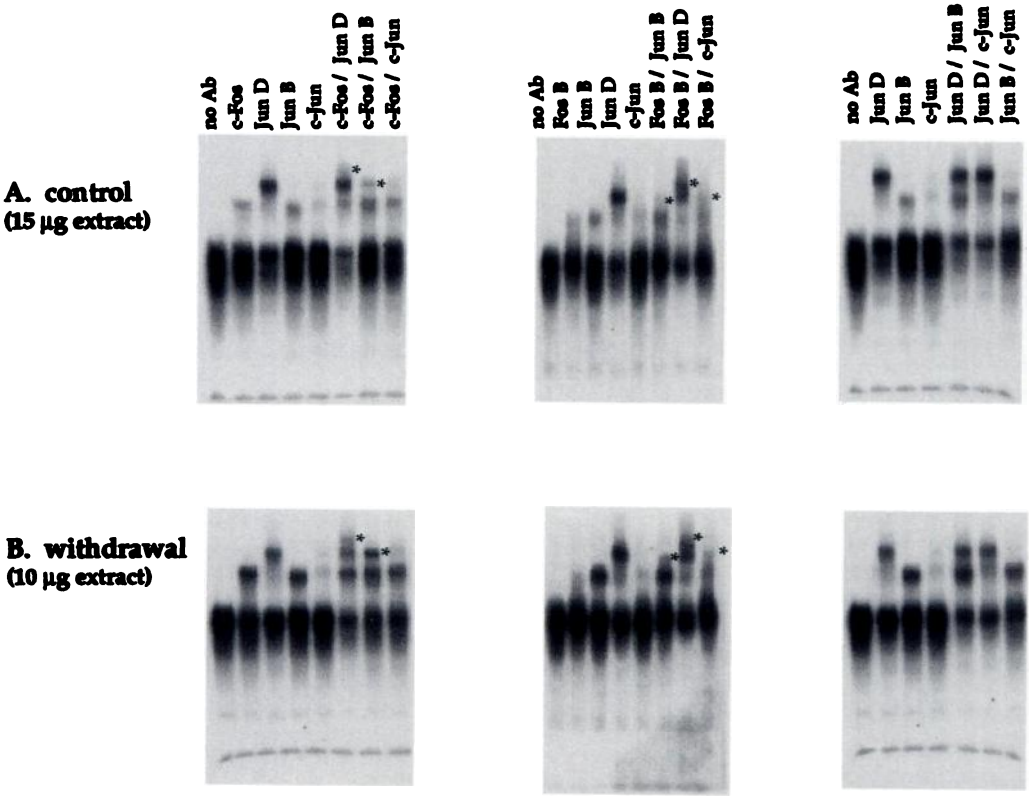

Fig. 9. Cortical AP-1 heterodimer levels are differentially regulated by precipitated morphine withdrawal. Incubation of the radiolabeled AP-1 oligonucleotide with cortical extracts prepared from control animals (placebo plus saline) ($n = 5$) (A) or from animals 2 hr after precipitated withdrawal (schedule 1 plus 100 mg/kg naloxone) ($n = 5$) (B) was followed by incubation with various combinations of polyclonal antisera directed against c-Fos, Fos-B, Jun-B, Jun-D, and c-Jun before gel electrophoresis. *, Presence of detectable AP-1 heterodimers. *no Ab*, no antibody added to the reaction.

mRNA expression. Circuits subserving sexual activity showed no altered pattern of c-*fos* mRNA expression after morphine withdrawal. A more detailed discussion is not possible here, but this preliminary analysis highlights the principle findings of this study.

The pattern of induced c-*fos* mRNA expression after precipitated opiate withdrawal partially overlapped with opiate receptor density. Whereas μ-, δ-, and κ-opiate receptors are differentially and extensively distributed throughout the rat brain (32, 33), the induced c-*fos* mRNA expression distribution was considerably smaller. Overlap occurred within the prefrontal cortex, neocortex, caudate putamen, olfactory tubercle, hippocampus, amygdala, ventral tegmentum, certain hypothalamic nuclei, locus ceruleus, and superior colliculi. No striking correlations between any particular receptor subtype and induced c-*fos* mRNA expression were apparent, although μ receptor densities showed the greatest degree of overlap with induced c-*fos* expression. There were areas in the rat brain, however, devoid of opiate receptors, such as the cerebellum, lateral habenula, and lateral geniculate, that exhibited induced c-*fos* mRNA expression, as well as regions with high opiate receptor density, such as the lateral amygdala and various thalamic nuclei (e.g., the reuniens, rhomboid, and ventromedial nuclei), that did not exhibit induced c-*fos* mRNA expression. The most parsimonious explanation of these data is that morphine dependence is not solely mediated by opiate receptors or opiate receptor-containing neurons.

Comparing neuronal metabolic activity during precipitated withdrawal (via assessment of 2-DG uptake, as detailed previously) (34, 35) with induced c-*fos* mRNA expression can provide insights regarding the specificity of transcriptional responses. Several brain areas where c-*fos* mRNA expression was increased after withdrawal also exhibited greater 2-DG uptake than did matched controls; these areas include the central nucleus of the amygdala, nucleus accumbens, medial and lateral septum, medial and lateral preoptic area, paraventricular nucleus of the hypothalamus, suprachiasmatic nucleus, lateral hypothalamic area, lateral habenula, and interpenduncular complex. The cortex, along with the pyramidal and granule cells of the hippocampus, showed increased c-*fos* mRNA expression after precipitated withdrawal but no increased 2-DG uptake. In contrast, the anterior medial and lateral nuclei of the thalamus, paratenial nucleus, and mammillary nucleus showed no increase in c-*fos* mRNA expression but a robust increase in 2-DG uptake after withdrawal. The relatively limited coincidence of increased c-*fos* mRNA expression and 2-DG uptake and the presence of 2-DG-positive and c-*fos*-negative regions as well as c-*fos*-positive and 2-DG-negative areas in the brain argue against c-*fos* as a sole marker of metabolic activity.

The functional relevance of transcriptional changes in *fos* and *jun* gene transcription was partially assessed by gel retention analysis of AP-1 DNA/protein complexes after morphine withdrawal. Precipitated morphine withdrawal produced an increase in AP-1 binding, an apparent increase in the level of specific Fos and Jun proteins present within these AP-1 complexes, and an apparent alteration of relative AP-1 heterodimer levels, in a brain region-specific fashion. Many of the increased changes in AP-1 binding presumably reflect changes at the transcriptional level. For example, increased c-Fos and Jun-B AP-1 binding is probably due to increased transcription of the c-*fos* and *jun*-B genes, respectively. However, increased Jun-D binding occurred in the absence of any observed changes at the mRNA level. It is uncertain how morphine withdrawal increased Jun-D binding within AP-1 complexes, although one study involving the proenkephalin promoter has shown a requirement for phosphorylation of Jun-D, regarding its ability

to enhance transcription (13). Indeed, the phosphorylation state of additional transcription factors has been shown to be fundamental to their function (14).

Precipitated withdrawal also increased the apparent relative levels of AP-1 heterodimers in a brain region-specific fashion. This observation suggests an expanded mechanism underlying brain region- and cell-specific AP-1-mediated alterations in the pattern of gene expression. Because functional genomic AP-1 sites are neither identical nor flanked by identical DNA sequences, the context of the AP-1 site becomes an issue; a specific Fos/Jun homo- or heterodimer could exhibit a greater preference for one genomic site versus another based on the sequence in which it is embedded (9). Added to this is the possibility of having distinct combinations of AP-1 heterodimers present and differentially regulated in a cell-specific fashion. Thus, the AP-1 system and its various Fos and Jun constituents can be regulated both transcriptionally and post-translationally in a cell-specific fashion after morphine withdrawal. Presumably, this results in altered patterns of expression of different target genes within those responsive cells.

Possible targets of dimerized Fos and Jun transcription factors are genes containing AP-1 sites at or near their promoter regions. Most of the studies to date regarding the effects of exogenous opiates on gene expression have focused on gene products related to the opioid receptor system, because it has been proposed that exogenous opioids perturb the endogenous opioid systems. Chronic morphine treatment has been shown by some to down-regulate proenkephalin mRNA levels in the striatum and prodynorphin mRNA levels in the striatum, hippocampus, and hypothalamus (36, 37). In contrast, other studies have failed to find any changes in hypothalamic expression of pro-opiomelanocortin, proenkephalin, and prodynorphin after a similar morphine administration paradigm (38). The proenkephalin gene has a functional AP-1 site within its promotor region and presumably represents a putative target of AP-1-regulated expression. It has also been reported to exhibit transcriptional regulation within the paraventricular nucleus of the hypothalamus after precipitated morphine withdrawal (39). Corticotrophin-releasing hormone gene expression was also shown to be increased in the paraventricular nucleus after withdrawal (40), and this effect could likewise be mediated through AP-1 sites within its promotor region. Other putative AP-1 target genes remain to be discovered.

By assessing the regulation of the AP-1 system at both transcriptional and semifunctional levels, this study suggests that there are AP-1-containing genes subject to transcriptional regulation during morphine withdrawal. Perhaps strategies to identify these target genes can be aided by knowledge of the types of AP-1 homo- and heterodimers present within specific brain regions. Morphine withdrawal produces a variety of complex behaviors whose molecular basis is poorly understood. This study suggests that there is a rich and complex matrix of transcriptional responses throughout the brain, which may underlie the neural plasticity associated with morphine dependence.

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