D1 Dopamine Receptors Modulate ΔFosB Induction in Rat Striatum after Intermittent Morphine Administration

Daniella L. Muller and Ellen M. Unterwald

Department of Pharmacology and Center for Substance Abuse Research, Temple University School of Medicine, Philadelphia, Pennsylvania (D.L.M., E.M.U.); and The Rockefeller University, New York, New York (E.M.U.)

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ABSTRACT

Induction of the transcription factor ΔFosB was studied to examine neurochemical adaptations produced by repeated opiate administration. The mechanism of this induction was also investigated. The 35- to 37-kDa isoforms of ΔFosB, also referred to as the chronic Fras, were measured in the nucleus accumbens, caudate putamen, and frontal cortex of male Sprague-Dawley rats after either an acute injection of morphine or an escalating dosing schedule of morphine for 10 days. Heroin was also tested to determine whether the findings extend to other opiates. Results from Western blot analysis using an anti-fosB antibody demonstrate that 10-day intermittent escalating dose morphine produced a significant increase in ΔFosB-immunoreactivity in the nucleus accumbens, caudate putamen and frontal cortex, whereas a single injection of morphine had no effect on Fra immunoreactivity. Heroin administered twice daily for 10 days by an intermittent escalating dose schedule also induced ΔFosB in the caudate putamen, but not in the nucleus accumbens or frontal cortex. Daily pretreatment with the selective D1-like dopamine receptor antagonist SCH 23390 [R-(-)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride] significantly blocked morphine-induced ΔFosB induction in the nucleus accumbens and caudate putamen, but not in the frontal cortex. These results demonstrate that morphine-induced ΔFosB up-regulation in the striatum, but not in the frontal cortex, is modulated by D1 dopamine receptors, suggesting that the mechanisms involved in the up-regulation of these chronic Fras by morphine is brain region-specific.

Alterations in brain neurochemistry after repeated drug exposure undoubtedly underlie the pathophysiology of addiction. It is thought that transcription factors triggered by acute and chronic exposure to opiates and other drugs of abuse initiate these changes at the cellular and molecular level by influencing gene expression (Nestler et al., 2001). A protein that has been studied for its involvement in the chronic effects of drugs of abuse is ΔFosB. This transcription factor, a product of the FosB gene, is derived from the C-terminal region of the FosB protein (Nakabeppu and Nathans, 1991). Upon induction of ΔFosB, the protein heterodimerizes with Jun family proteins (Gentz et al., 1989; Nakabeppu and Nathans, 1991), subsequently forming an activator protein-1 binding complex, which can alter the expression of other genes such as those encoding the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunit, GluR2, dynorphin and cyclin-dependent kinase-5 (Cdk5) (Chen et al., 1997; Kelz et al., 1999; Bibb et al., 2001). Therefore, drugs of abuse, through activation of these target genes by ΔFosB can produce long-term changes in brain neurochemistry.

Using an inducible expression system in vitro, Chen et al. (1997) showed that highly stable isoforms of ΔFosB (referred to as chronic Fra or Fos-related antigens) are derived from the ΔFosB protein and furthermore, that these chronic Fras display a unique time course of activation different from that of ΔFosB. A number of reports have concurred that although ΔFosB is induced after acute drug exposure, stable isoforms of this protein are observed after chronic drug intake and display a longer half-life (Hope et al., 1994; Nye et al., 1995; Nye and Nestler, 1996). For example, Nestler and colleagues outlined the temporal pattern of activation of cocaine-induced ΔFosB up-regulation and found that these proteins accumulate over a 7-day period and persist for up to a week after cessation of cocaine administration (Hope et al., 1994). Likewise, ΔFosB levels are significantly induced in the stri-

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor; Cdk5, cyclin-dependent kinase-5; SCH 23390, R-(-)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; ANOVA, analysis of variance; MK-801, dizocilpine maleate; GYKI-52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride.
at a high level after 5-day continuous morphine pellet implantation, but not after acute morphine administration (Nye and Nestler, 1996).

There are interactions between dopaminergic and opioidergic systems in the central nervous system. For example, morphine indirectly acts on the dopaminergic system (Johnston and North, 1992). Morphine activates μ opioid receptors located on GABA-containing interneurons in the substantia nigra and ventral tegmental area, brain regions rich in dopamine-containing cell bodies. This action disinhibits dopamine neurons in the substantia nigra and ventral tegmental area, thereby increasing the release of dopamine in the caudate putamen and nucleus accumbens, respectively (Iwatsubo and Clouet, 1977; Di Chiara and Imperato, 1988).

Both the mesolimbic and nigrostriatal dopamine pathways are principal areas in the brain that mediate drug reward (Koob, 1992) and addictive-related behaviors (Graybiel et al., 1990). The mesocortical dopaminergic pathway is another circuit in the brain that is involved in drug addiction (for review, see Steketee, 2003). Dopaminergic neurons in the midbrain ventral tegmental area project primarily to pyramidal neurons in the prefrontal cortex. These neurons then provide feedback to subcortical neurons in the ventral tegmental area and limbic areas of the brain. According to Goldstein and Volkow (2002), it is the interplay of the mesocortical and mesolimbic dopaminergic circuits that ultimately influences the compulsive and motivational properties of drug addiction (Goldstein and Volkow, 2002).

Evidence suggests that the dopaminergic system participates in morphine-induced regulation of gene expression (Liu et al., 1994; Bontempi and Sharp, 1997). Morphine acutely induces cFos expression in the nucleus accumbens and caudate putamen, and this is blocked by pretreatment with the D1-like dopamine receptor antagonist SCH 23390 (Liu et al., 1994; Bontempi and Sharp, 1997). It has also been shown that the induction of ΔFosB by psychostimulants is mediated by the D1 dopamine receptor pathway. ΔFosB up-regulation in the nucleus accumbens and caudate putamen by cocaine and amphetamine is attenuated by SCH 23390 (Nye et al., 1995). Therefore, since D1 dopamine receptors are involved in the regulation of ΔFosB by psychostimulants and morphine indirectly activates D1 dopamine receptors, we hypothesize that D1 dopamine receptors are involved in morphine-induced ΔFosB up-regulation. Thus, the present study examined whether pretreatment with the D1-like dopamine receptor antagonist would alter ΔFosB levels after intermittent morphine administration. The regulation of ΔFosB by a single injection of morphine as well as chronic heroin administration was also characterized.

Materials and Methods

Animals. Male Sprague-Dawley (200–250 g; Harlan, Indianapolis, IN) rats were housed in groups of two per cage in a standard animal facility for 5 to 7 days before the start of the experiments (12-h light/dark cycle; lights on 7:00 AM). All rats were weighed daily and had ad libitum access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Acute Morphine Administration. A single s.c. saline injection was given to each rat 48 h before the start of the acute morphine studies to acclimate the animals to the injection procedure. Rats received a single injection of morphine sulfate (50 mg/kg, s.c.) or saline (1 ml/kg, s.c.) and were euthanized 4 h postinjection.

Chronic Morphine/Heroin Administration. In the chronic morphine studies, rats were administered morphine sulfate or heroin (both generously supplied by the National Institute on Drug Abuse, Rockville, MD) in an escalating dosing schedule that consisted of twice-daily s.c. injections for 10 days (injections were given at 9:00 AM and 6:00 PM). This intermittent dosing paradigm has been shown to induce sensitization to the acute locomotor-activating effects of morphine and has been used as an animal model to investigate addictive processes (for review, see Spanagel, 1995). All injections were given in a volume of 1 ml/kg body weight. Table 1 shows the dosing schedules for morphine and heroin.

For the antagonist study, four groups of rats received intermittent escalating dose morphine or saline for 10 days as described above. In addition, an i.p. injection of the D1-like dopamine receptor antagonist SCH 23390 (1 mg/kg; Sigma-Aldrich, St. Louis, MO) or saline was given 30-min before each morphine or saline injection. Animals were euthanized 4 h after the last injection. SCH 23390 exhibits a 670-fold higher selectivity for D1 than D2 dopamine receptors (Hytell, 1983). This ligand has equal affinity at both D1 and D5 dopamine receptors (for review, see Bourne, 2001).

Preparation of Brain Extracts and Immunoblotting Procedure. After drug administration, rats were euthanized by brief exposure to CO2 and decapitated in an unconscious state. The nucleus accumbens, caudate putamen and frontal cortex were carefully and rapidly dissected on ice as described in Glowinski and Iversen (1966). Each brain region was either immediately sonicated in boiling 1% SDS buffer and boiled for 5 min or homogenized in an electrophoretic mobility shift assay buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 20% glycerol, 5 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 5 mM dithiothreitol, 0.5 mM p-aminobenzoamide, 10 μg/ml leupeptin, and 1 μg/ml pepstatin) and then centrifuged at 13,000 rpm for 10 min. The sample was then aliquotted and frozen at –80°C until assayed. Similar inductions of chronic Fras after morphine administration were obtained with both tissue extraction procedures. Protein concentrations were determined using the Lowry method. Protein extracts (50 μg) were then subjected to SDS-polyacrylamide gel electrophoresis (10% Tris-HCl Bio-Rad Ready-gels; Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Equal protein loading was visualized using the general protein stain Ponceau S. Membranes were blocked for 1 h in blocking buffer, containing 5% nonfat dry milk and Tris-buffered saline/Tween 20 and then incubated overnight in an anti-FosB antibody (1:1000; catalog no. sc-48; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All blots were also incubated for 90 min in an anti-tubulin antibody (1:50000; catalog no. T8535; Sigma-Aldrich) to ensure that there were no differences in protein loading or transfer. After washes in Tris-buffered saline/Tween 20, the membranes were incubated in either anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase (1:5000; Vector Laboratories, Burlingame, CA) for 1 h. Immunoreactivity was detected by chemiluminescence using Supersignal West Pico chemiluminescent substrate (Pierce Chemical, Oakbrook Terrace, IL).
activity was evident in the nucleus accumbens (115 versus heroin, respectively). Significance was set at \( p < 0.05 \).

**Results**

**ΔFosB Immunoreactivity in the Nucleus Accumbens, Caudate Putamen, and Frontal Cortex Is Up-Regulated after 10-Day Morphine Administration.** ΔFosB levels in the nucleus accumbens, caudate putamen, and frontal cortex were not induced by a single morphine injection (Student’s \( t \) test; all \( p \) values > 0.05; \( n = 6–7 \)/group; Fig. 1). The chronic isoforms of ΔFosB migrate as 35- to 37-kDa protein bands and are present in higher amounts after repeated drug treatment. Unlike acute morphine, intermittent escalating dose morphine administration (see Table 1 for morphine dosing schedule) in rats significantly induced the chronic isoforms of ΔFosB in the nucleus accumbens, caudate putamen, and frontal cortex (Student’s \( t \) test; all \( p \) values < 0.05; \( n = 8 \)/group; Fig. 2, a–c). As reported previously (Nye et al., 1995; Kelz et al., 1999; Ehrlich et al., 2002), two bands of ΔFosB (35–37 kDa) were detected in tissue from the nucleus accumbens, whereas only one band was seen in the caudate putamen and frontal cortex.

**ΔFosB Immunoreactivity in the Caudate Putamen, but Not in the Nucleus Accumbens or Frontal Cortex, Is Altered by Chronic Heroin Administration.** To date, ΔFosB induction has only been reported after a single opiate, morphine (Nye and Nestler, 1996). It was of interest to examine whether the findings with morphine extend to other opiates, particularly heroin, given its high abuse potential. Animals received twice-daily injections of heroin or saline for 10 days. The initial dose was 0.5 mg/kg, escalating every 2 days reaching a final dose of 8 mg/kg on day 10. Figure 3 illustrates the effects of chronic heroin administration on ΔFosB immunoreactivity in the nucleus accumbens, caudate putamen, and frontal cortex. A Student’s \( t \) test revealed that the levels of ΔFosB isoforms in the caudate putamen were significantly higher compared with saline-injected animals (159 ± 21% of control; \( p = 0.048 \); \( n = 8 \)/group; Fig. 3b), whereas no significant differences in chronic Fra immunoreactivity was evident in the nucleus accumbens (115 ± 22% of control; \( p > 0.05 \); Fig. 3a) or in the frontal cortex (133 ± 29% of control; \( p > 0.05 \); Fig. 3c).

**SCH 23390 Pretreatment Blocks Morphine-Induced Up-Regulation of ΔFosB in the Nucleus Accumbens and Caudate Putamen but Not in the Frontal Cortex.** To investigate whether morphine-induced up-regulation of ΔFosB in the nucleus accumbens, caudate putamen and frontal cortex is mediated by activation of D1 dopamine receptors, the selective D1-like dopamine receptor antagonist, SCH 23390 (1 mg/kg, i.p.) or saline (1 ml/kg, i.p.), was given 30 min before twice-daily morphine or saline. Figure 4 illustrates mean (±S.E.M.) ΔFosB immunoreactivity in the nucleus accumbens, caudate putamen, and frontal cortex across the four treatment groups. A two-way ANOVA with drug treatment \( \times \) pretreatment factors revealed a significant main effect for the drug treatment groups in the nucleus accumbens \( F(1,51) = 9.343; p < 0.0036 \); \( n = 13–16 \)/group; Fig. 4a), caudate putamen \( F(1,55) = 9.683; p < 0.0029 \); Fig. 4b, and frontal cortex \( F(1,55) = 22.87; p < 0.0001 \); Fig. 4c. In the nucleus accumbens and caudate putamen, Bonferroni’s post hoc assessments revealed that rats given chronic morphine displayed significantly higher levels of Fra immunoreactivity than saline-injected control animals (\( p \) values < 0.01). Fra immunoreactivity was not significantly induced in animals that received SCH 23390 followed by morphine. In the frontal cortex, levels of ΔFosB were significantly higher after morphine administration compared with saline-injected control animals (\( p \) < 0.01). Similarly, animals pretreated with SCH 23390 followed by morphine displayed higher levels of ΔFosB compared with control animals (\( p < 0.01 \)), suggesting that pretreatment with SCH 23390 did not alter the up-regulation of these chronic Fras by morphine in the frontal cortex.

**Discussion**

It is well established that the transcription factor ΔFosB accumulates in the brain as a result of a variety of chronic stimuli, including drugs of abuse (Hope et al., 1994; Nye et al., 1995; Rodriguez et al., 2001; Ehrlich et al., 2002). An earlier report by Nye and Nestler (1996) demonstrates that chronic morphine treatment induces ΔFosB in specific brain regions, an effect that is blocked by pretreatment with the opioid receptor antagonist naltrexone. The present study set out to further characterize the induction of ΔFosB by opiates as well as examine whether indirect activation of D1 dopamine receptors is involved in its induction. Results from the
present study demonstrate that 10-day intermittent morphine administration significantly increased levels of ΔFosB in the nucleus accumbens, caudate putamen, and frontal cortex, an effect not seen after a single morphine injection. Chronic Fra immunoreactivity was significantly elevated in the caudate putamen, but not in the nucleus accumbens or frontal cortex after repeated administration of another opiate, heroin. Heroin is rapidly metabolized to morphine and,
that the pharmacological profiles of morphine and heroin may differ (Rady et al., 1991; Rossi et al., 1996). For example, CXBK mice that are insensitive to morphine, maintain their sensitivity to heroin-induced analgesia (Rossi et al., 1996). Thus, the distinct pharmacological profiles of these drugs may account for the differential patterns of chronic Fra induction by morphine and heroin. It is important to note that only a single dosing paradigm of heroin was tested in the present study; therefore, it is difficult to determine whether higher doses of heroin would have induced ΔFosB in other brain regions similar to morphine. Additional studies are needed to resolve this issue.

The morphine-induced up-regulation of ΔFosB in the nucleus accumbens and caudate putamen was partially blocked by pretreatment with SCH 23390, a selective D1-like dopamine receptor antagonist. The high levels of ΔFosB immunoreactivity in the frontal cortex after morphine were not altered by pretreatment with SCH 23390. Together, these results suggest that the up-regulation of ΔFosB in the nucleus accumbens and caudate putamen by morphine is modulated by activation of D1 dopamine receptors; however, D1 dopamine receptors do not seem to be involved in morphine-induced ΔFosB up-regulation in the frontal cortex.

μ-Opioid receptor agonists indirectly increase dopamine release in the nucleus accumbens and caudate putamen (Di Chiara and Imperato, 1988). Dopamine’s actions are mediated through dopamine receptors (D1-like and D2-like), members of the G protein-coupled receptor family. Differential D1 dopamine receptor distribution in the brain may explain the findings of differential involvement of D1 dopamine receptors in morphine-induced ΔFosB up-regulation among the various brain areas. D1 dopamine receptors are expressed at much lower levels in the frontal cortex than in the caudate putamen and nucleus accumbens (Boyson et al., 1986). Diop et al. (1988) showed that the density of \[^3H\]SCH 23390 binding is 10 to 20 times greater in the striatum than in the cortex. The present study found that morphine-induced ΔFosB up-regulation was attenuated by SCH 23390 in both the nucleus accumbens and the caudate putamen, but not in the frontal cortex. Therefore, it is possible that this is due to the small number of D1 dopamine receptors located in the frontal cortex compared with that in the nucleus accumbens or caudate putamen. As stated above, SCH 23390 binds to both D1 and D5 dopamine receptors with \(K_i\) values of 0.2 and 0.3 nM, respectively (for review, see Bourne, 2001). Therefore, it is not possible to determine which receptor subtype is responsible for the induction of ΔFosB by morphine. It is likely, however, that D1 dopamine receptors are primarily mediating this effect since D5 dopamine receptor mRNA, although present in these areas, is found at much lower levels than D1 dopamine receptor mRNA (Tiberi et al., 1991; Meador-Woodruff et al., 1992). For example, Tiberi et al. (1991) used in situ hybridization to map D5 dopamine receptor mRNA in rat brain. They found little D5 dopamine receptor mRNA in striatum, an area where D1 dopamine receptor mRNA is abundant.

Although our data indicate that D1 dopamine receptors are important for morphine-induced ΔFosB up-regulation in the striatum, other receptor systems also may be involved. For example, MK-801, an \(N\)-methyl-\(d\)-aspartate antagonist, blocks the induction of cFos and junB by morphine in the striatum (Liu et al., 1994). Furthermore, morphine-induced
Likewise, chronic haloperidol administration induces Fra in the limbic regions of the brain (Nye et al., 1995). Dopamine receptor antagonist eticlopride induces chronic contrast to D1 dopamine receptors, D2 dopamine receptors result in activation of both D1 and D2 dopamine receptors. In Bontempi and Sharp, 1997).

Expression by morphine in these same studies (Liu et al., 1994; Bontempi and Sharp, 1997).

Dopamine release stimulated by morphine is likely to result in activation of both D1 and D2 dopamine receptors. In contrast to D1 dopamine receptors, D2 dopamine receptors play a unique role in the regulation of Fos proteins. The D2 dopamine receptor antagonist eticlopride induces chronic Fra in the limbic regions of the brain (Nye et al., 1995). Likewise, chronic haloperidol administration induces ΔFosB in the rat striatum. This is consistent with other reports that have demonstrated increases in Fra immunoreactivity after antipsychotic treatment (Rodriguez et al., 2001a,b). Elevated levels of ΔFosB protein were also observed in postmortem striatal tissue of patients with Parkinson’s disease (Tekumalla et al., 2001). Whether the increase in ΔFosB was a result of dopaminergic agonist treatment or dopamine denervation was not assessed; however, both have been shown to induce ΔFosB in the striatum (Hope et al., 1994). Although there is still much unknown about the physiological significance of increased levels of ΔFosB in the striatum after chronic manipulations, it is clear that the dopaminergic system plays an integral role in the regulation of ΔFosB by not only drugs of abuse but also drug treatments for a number of central nervous system disorders.

Little is known regarding the role of ΔFosB in modulating the behavioral effects of morphine; however, over the past decade, the role of ΔFosB in modulating cocaine-induced behaviors has been investigated. A number of studies using genetically manipulated mice have shown that cocaine-induced behavioral responses are modulated by ΔFosB (Hiroi et al., 1997; Kelz et al., 1999; Haile et al., 2001). For example, transgenic mice overexpressing ΔFosB in the striatum show exaggerated behavioral responses to chronic cocaine (Kelz et al., 1999; Colby et al., 2003). Both cocaine-induced locomotor activity and conditioned place preference are enhanced in these mice (Kelz et al., 1999). Additionally, overexpressing ΔFosB transgenic mice self-administer lower doses of cocaine than control mice (Colby et al., 2003). Together, these studies suggest that the transcription factor, ΔFosB, plays a facilitative role in modulating cocaine-induced behaviors. Thus far, the behavioral studies have been limited to cocaine; therefore, the role of ΔFosB in modulating morphine-induced behaviors has yet to be elucidated.

Specific target genes for ΔFosB have been identified that give insight into the potential functional significance of this protein in regard to addiction. A number of reports have identified cellular proteins such as GluR2 and Cdk5, which are elevated in inducible transgenic mice overexpressing ΔFosB (Kelz et al., 1999; Bibb et al., 2001). There is also evidence to suggest that these same genes may be involved in ΔFosB regulation by morphine. Morphine-induced cFos expression in the striatum is attenuated by the AMPA receptor antagonist, GYKI-52466 (Garcia et al., 2003). The development of morphine antinoceptive tolerance is attenuated after intrathecal administration of roscovitine, a Cdk5 inhibitor (Wang et al., 2004). In another report, acute morphine administration increases the levels of Cdk5 in rat cortex, whereas a 40% decrease in Cdk5 protein levels is seen after 5-day escalating dose morphine administration (Ferrer-Alcon et al., 2003). Further studies are needed to examine not only the role of GluR2 and Cdk5 in ΔFosB induction by morphine but also to identify other targets that may be involved in opioid-mediated signaling in vivo.

It seems that the type of dosing schedule (i.e., continuous versus intermittent) yields differences in ΔFosB induction. Animals receiving chronic continuous cocaine show little or no induction of ΔFosB in the striatum compared with the robust response seen in animals receiving intermittent cocaine (Nye et al., 1995). Likewise, a previous report failed to show a significant induction of Fras in the frontal cortex after continuous morphine treatment (Nye and Nestler, 1996), whereas our results show a 50% increase in Fra immunoreactivity in the frontal cortex after intermittent morphine administration. Similarly, Torres and Rivier (1992) showed that administration of cocaine via continuous exposure has no effect on cFos expression, whereas cFos expression is induced in the caudate putamen after intermittent cocaine administration.

In summary, the present findings demonstrate that morphine-induced ΔFosB induction in the striatum, but not in the frontal cortex, is indirectly modulated by D1 dopamine receptors. The mechanism by which these chronic Fra are induced in the frontal cortex is unclear; however, further investigation into the potential pathways mediating ΔFosB in this brain region as well as others is important for a more comprehensive understanding of this transcription factor’s role in the regulation of gene expression produced by repeated morphine administration. Identifying the brain region specific intracellular pathways that mediate ΔFosB induction by morphine is necessary to reveal potential downstream cellular targets. Identification of these target genes will ultimately provide insight into the functional significance of the chronic Fra in morphine action as well as their relevance to opiate tolerance, dependence, and addiction.

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