Neurofilament Proteins and the Mesolimbic Dopamine System: Common Regulation by Chronic Morphine and Chronic Cocaine in the Rat Ventral Tegmental Area

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The ventral tegmental area (VTA) and its dopaminergic projections appear to mediate some of the rewarding properties of opiates, cocaine, and other drugs of abuse. In a previous study, we demonstrated that chronic morphine and cocaine exert common actions on tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, in this dopaminergic brain reward region (Beitner-Johnson and Nestler, 1991). In the present study, we investigated the effects of chronic morphine and cocaine on other phosphoproteins in the VTA by back phosphorylation and two-dimensional electrophoretic analysis. It was found that a number of phosphoproteins, in addition to tyrosine hydroxylase, were regulated similarly by the two drug treatments in this brain region. Several of these morphine- and cocaine-regulated phosphoproteins were identified as neurofilament (NF) proteins. Chronic, but not acute, administration of either morphine or cocaine was found to decrease levels of the three NF proteins, NF-200 (NF-H), NF-160 (NF-M), and NF-68 (NF-L), by between 15% and 50% in the VTA by back phosphorylation, immunolabeling, and Coomassie blue staining. Such regulation of NF proteins was selective, in that no detectable changes were observed in the levels of eight other major cytoskeletal or cytoskeletal-associated proteins analyzed. Furthermore, NF levels were not altered by chronic treatment with either imipramine or haloperidol, two psychotropic drugs without reinforcing properties, or by chronic stress. Morphine and cocaine regulation of NFs showed regional specificity, as NF levels were not altered in the substantia nigra, or other parts of the brain or spinal cord, by these drug treatments. NFs are thought to function as determinants of neuronal morphology and to be associated with axonal transport. Thus, decreased NF levels in the VTA in response to chronic morphine and chronic cocaine could lead to drug-induced alterations in the structural and functional properties of this brain region, which may represent, in turn, part of a common biochemical basis of morphine and cocaine addiction and craving.

The reinforcing properties of drugs of abuse are important components of their addiction liability. Several lines of evidence, based initially on animal self-administration studies, have focused attention on dopaminergic neurons in the ventral tegmental area (VTA) and certain of its projection regions, most notably the nucleus accumbens (NAc), as neural substrates mediating the reinforcing properties of several types of drugs of abuse (for reviews, see Fibiger, 1978; Bozarth, 1986; Wise and Bozarth, 1987; Koob and Bloom, 1988; Liebman and Cooper, 1989). A number of studies have reported that lesions of the VTA and/or NAc attenuate opiate, cocaine, and amphetamine self-administration and conditioned place preference (Lyness et al., 1979; Roberts and Koob, 1982; Spyraki et al., 1983; Pettit et al., 1984; Zito et al., 1985; Bozarth and Wise, 1986; Carr et al., 1989). In addition, virtually all drugs abused by humans, including opiates, cocaine, amphetamine, ethanol, nicotine, and tetrahydrocannabinol, increase dopamine release in the NAc when administered systemically to rats (DiChiara and Imperato, 1988; Chen et al., 1990). These studies identify common acute actions of drugs of abuse on the mesolimbic VTA-NAc pathway that may contribute to their rewarding properties, and implicate the VTA and the NAc as brain regions involved in a common mechanism of drug addiction. Since these addictive drugs have diverse primary sites of action (e.g., distinct cell surface receptors), indirect actions of these drugs on intracellular proteins may be a convergence point at which various drugs of abuse exert similar effects on mesolimbic dopamine function.

Drugs of abuse also have profound chronic effects on brain function. There is growing evidence in animals and in people that chronic use of opiates and psychostimulants increases drug craving (Lett, 1989; Piazza et al., 1989; Jaffe, 1990). Moreover, chronic exposure to these drugs leads to similar types of sensitization to their acute locomotor-activating effects (Babbini and Davis, 1972; Post and Rose, 1976; Kilbey and Ellinwood, 1977). A number of studies support the view that these chronic actions also appear to be mediated, at least in part, via the VTA-NAc pathway (Kalivas and Duffy, 1988; Koob and Bloom, 1988; Henry et al., 1989; Pettit et al., 1990; Stekatee et al., 1991), although the precise biochemical and molecular mechanisms involved remain uncertain.

In previous studies, we have shown that chronic morphine and chronic cocaine treatments produce some similar effects on levels of G-proteins, adenylate cyclase, and cAMP-dependent protein kinase in the mesolimbic dopamine system (Nestler et al., 1990b; Terwilliger et al., 1991a). In addition, we have found that chronic morphine and cocaine treatments similarly regulate

tyrosine hydroxylase in these brain regions (Beitner-Johnson and Nestler, 1991). In the present study, we furthered these investigations by examining the effects of chronic morphine and chronic cocaine on protein phosphorylation in the VTA by use of a back-phosphorylation procedure. Back phosphorylation is a well-established procedure with which to study brain phosphoproteins, and provides information about the total levels and phosphorylation state of individual phosphoproteins (Nestler and Greengard, 1984, 1989). Given the important role of protein phosphorylation in the regulation of neuronal function, such studies offer an approach by which to reveal the types of proteins and neuronal processes altered by morphine and cocaine treatment.

We report here the identification of several MCRPPs (morphine- and cocaine-regulated phosphoproteins) and show that MCRPP-200, -160, -68, and -64 are neurofilament (NF) proteins. NFs are neural-specific intermediate filaments that appear to be components of the neuronal cytoskeleton associated with the determination of neuronal morphology and, possibly, axonal transport (Hoffman and Lasek, 1975; Tytell et al., 1981; Hoffman et al., 1984; Hall et al., 1991). NFs are found in all, or virtually all, neurons in the CNS and PNS (Robinson and Anderton, 1988). The finding that these proteins, like tyrosine hydroxylase, are similarly altered in the VTA by chronic morphine and chronic cocaine suggests that these drug treatments induce common functional changes within the VTA. Possible functional sequelae mediated via such drug regulation of NFs, which could represent part of a general biochemical mechanism underlying opiate and cocaine addiction and craving, are discussed.

Materials and Methods

In vivo drug treatments. Male Sprague–Dawley rats (initial weight, 140–180 gm; obtained from CAMM, Wayne, NJ) were used in these studies. Chronic morphine was administered as described previously (Guitart and Nestler, 1989). Briefly, rats received one 75 mg pellet of morphine base (NIDA) subcutaneously under light halothane anesthesia daily for 5 d, and experiments were performed on day six. In initial studies, control rats were implanted with equivalent placebo pellets (NIDA). As in previous studies (Guitart and Nestler, 1989), we found that placebo pellets produced no changes in any phosphoproteins as compared to naive control rats; hence, subsequent experiments were performed using naive animals as controls. For chronic cocaine studies, rats were injected with cocaine hydrochloride (15 mg/kg; Sigma) intraperitoneally in 0.9% saline twice daily for 14 d. Control rats received equivalent saline injections. In most experiments, the animals were used on day 15, 30 min after the last A.M. injection. In some experiments, the animals were used on day 15, ~16 hr after the previous P.M. injection. Similar results were obtained under these two treatment conditions. These treatment protocols are similar to those used in previous studies, where they have been shown to induce states of opiate tolerance and dependence in the case of morphine (Blasig et al., 1973; Aghajanian, 1978; Rasmussen et al., 1990), and behavioral sensitization as well as electrophysiological and neurochemical alterations in mesolimbic dopamine neurons in the case of cocaine (Kilbey and Ellinwood, 1977; Henry et al., 1989; Lett, 1989; Brock et al., 1990; Pettit et al., 1990; Beitner-Johnson and Nestler, 1991; Ng et al., 1991). For chronic imipramine treatment, rats received intraperitoneal injections of imipramine (15 mg/kg; Sigma) once daily for 18 d. Control animals received saline injections, and rats were used on day 19. This administration protocol has been shown in previous studies to lead to chronic effects of imipramine on noradrenergic neurons of the locus coeruleus (LC; Nestler et al., 1990a). For chronic haloperidol treatment, rats were administered 1 mg/kg haloperidol orally (in the drinking water) for 21 d and were used on day 22. This protocol has been shown to induce depolarization inactivation of midbrain dopaminergic neurons (Chiodo and Bunney, 1983).

Chronic cold stress. Rats were housed three per cage in wire mesh cages (35 × 20.5 × 17 cm) in a colony room for at least 7 d prior to the start of the experiment. The room was maintained on a 12 hr light/12 hr dark cycle, and rats were allowed access to food and water ad libitum. Control rats remained in the colony room throughout the study. Rats exposed to cold stress were housed singly in plastic cages (28 × 15 × 15 cm) in a cold room maintained at 3–5°C for 5 d exactly as described by Melia et al. (1992). Rats were used in the morning of day 6.

Isolation of brain regions. Brains were removed rapidly from decapitated rats and cooled immediately in ice-cold buffer containing 126 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM D-glucose, pH 7.4. The VTA, substantia nigra (SN), and LC were obtained from 1 mm coronal cross sections using a McIlwain tissue slicer. Nuclei were excised from the slices using a 15 gauge syringe needle as described previously (Nestler and Tallman, 1988; Palkovits and Brownstein, 1988); bilateral punches (about 1.5 mg wet weight) were pooled from individual rats. The spinal cord and other brain regions were removed by gross dissection.

Back-phosphorylation assays, two-dimensional electrophoresis, and peptide mapping. Samples were prepared for direct back phosphorylation as described previously (Guitart and Nestler, 1989) with minor modifications. Briefly, brain punches were sonicated using a Kontes cell disrupter in 100 μl of an ice-cold buffer containing 20 mM citric acid and 0.01% Nonidet P-40, followed by the addition of another 100 μl of the same buffer. This method permitted easy sonication in a small volume, with a final pH of 2.8–3.0. The acidified homogenates were centrifuged at 10,000 × g in a Savant microfuge for 15 min at 4°C. The pellets were discarded and the supernatants ("acid extracts") were neutralized to pH 6.0–6.3 by the addition of 300 μl/ml of 200 mM Na_2HPO_4.

Back phosphorylation was performed as described previously (Guitart and Nestler, 1989), using purified catalytic subunit of cAMP-dependent protein kinase (Sigma) and γ-^{32}P-ATP (30 Ci/mmol; New England Nuclear). Final protein concentrations were typically 0.5–1.0 μg/μl, and equivalent amounts of back-phosphorylated extracts (containing 5–20 μg of protein) were subjected to two-dimensional gel electrophoresis as described (O'Farrell, 1975; Guitart and Nestler, 1989), with two modifications made in the isoelectric focusing protocol. First, LKB ampholines were substituted for Bio-Rad ampholines, as the former were found to be more stable and to produce better resolution in the isoelectric focusing dimension. These were used in the same ratio as described previously (4% ampholines, 1:1 pH 3.5–10:pH 5–7). Second, piperazine diacrylamide (Bio-Rad) was substituted for bisacrylamide as a cross-linking agent in the first dimension on a gram-for-gram basis. This was found to increase the strength and ease of handling of the tube gels, without altering the relative migration of individual phosphoproteins.

Identity between phosphoproteins identified on one- and two-dimensional gels by back phosphorylation was studied by one-dimensional peptide mapping analysis as described by Cleveland et al. (1977). Bands of individual phosphoproteins were excised from dried one- or two-dimensional gels and reswelled in 20 mM Tris pH 6.7, 0.1% SDS for 10 min at room temperature. The gel bands were then loaded onto standard one-dimensional SDS-polyacrylamide gels except that the stacking gels were 6 cm long and the resolving gels contained 15% acrylamide/0.6% bisacrylamide. *Staphylococcus aureus* V-8 protease (2.5 μg; Miles) was then loaded on top of the bands, and the gels were electrophoresed at 60 V overnight. Resulting gels were dried and autoradiographed.

Quantitation of back-phosphorylation data. Phosphorylated proteins were detected by autoradiography at room temperature for 12–72 hr. In each experiment, four control and four drug-treated rats were used, and each brain sample was analyzed in duplicate (i.e., duplicate back phosphorylation and two-dimensional gels). The amount of ^{32}P incorporated into individual bands was quantitated using 60 min scans of dried gels on a Betascope blot analyzer (Betagen). Back-phosphorylation levels of individual proteins derived from drug-treated rats were calculated as a percentage of the average levels from control rats.

Immunolabeling of neurofilaments and other proteins. Tissue samples were homogenized (10 mg wet weight/ml) in 2% SDS, and protein levels were determined using the method of Lowry et al. (1951). For one-dimensional immunoblotting, samples were adjusted to contain final concentrations of 50 mM Tris pH 6.7, 4% glycerol, 4% SDS, and 2% 2-mercaptoethanol, with bromophenol blue as a marker, and then boiled for 2 min. Aliquots (containing 10–40 μg protein) were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis with 6% acrylamide/0.24% bisacrylamide in the resolving gels. For two-dimensional immunoblotting, gel samples were prepared and electrophoresed exactly as described (O'Farrell, 1975; Guitart and Nestler, 1989), except that 100–150 μg of protein were loaded onto gels, and 0.03% SDS and
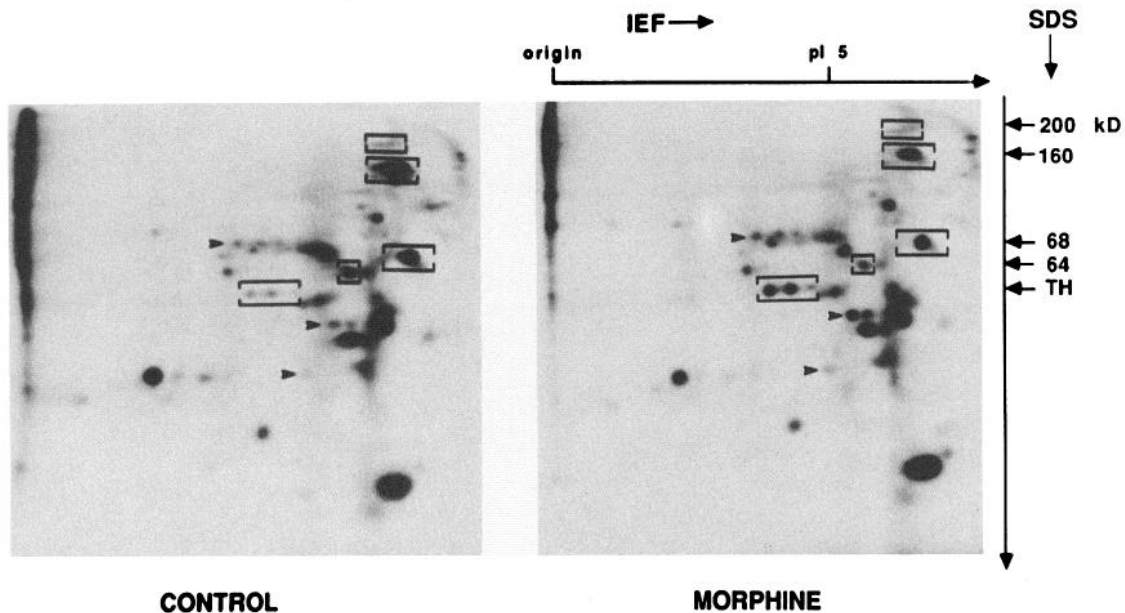
CONTROL　　　　　**MORPHINE**

Figure 1. Autoradiograms of two-dimensional gels illustrating the effect of chronic morphine on back phosphorylation in the VTA. Rats were treated chronically with morphine as described in Materials and Methods. Isolated VTA nuclei from control and drug-treated rats were then subjected to back phosphorylation with cAMP-dependent protein kinase and γ-^{32}P-ATP and to two-dimensional gel electrophoresis, as described. Resulting gels were dried and autoradiographed. *Brackets* indicate the positions of phosphoproteins of M_r 200, 160, 68, and 64 whose back phosphorylation is decreased by both chronic morphine and chronic cocaine (see Fig. 2), and also indicate the position of a 58 kDa phosphoprotein [known to be tyrosine hydroxylase (*TH*)] whose back phosphorylation is increased by chronic morphine and chronic cocaine. Several other unknown phosphoproteins of 71, 51, and 39 kDa (indicated by *arrowheads*), which are not studied further here, also appeared to be regulated by morphine and cocaine.

4% Nonidet P-40 (final concentrations) were included in the isoelectric focusing dimension to facilitate sample solubilization. Proteins were transferred electrophoretically from one- and two-dimensional gels to nitrocellulose papers. When immunolabeling NF-200 and NF-160, 0.025% SDS was included in the transfer buffer, as this was found to increase the efficiency of the electrophoretic transfer process. Nitrocellulose papers were then incubated with 2% nonfat dry milk (or 1% goat serum; GIBCO, in the case of the SMI-31 antibody) in a buffer containing 10 mM sodium phosphate, pH 7.2, 140 mM NaCl, and 0.05% Tween 20 (Sigma). NFs were then immunolabeled using the following antibodies: anti-NF-200 (clone N52, 1:1000; Sigma), anti-NF-160 (clone NN18, 1:750; Sigma), anti-NF-68 (clone NR4, 1:500; Boehringer Mannheim), anti-NF-66 (1:10,000; kindly provided by Dr. F.-C. Chiu, Albert Einstein College of Medicine), and anti-highly phosphorylated NF-200/160 (clone SMI-31, 1:5000; Sternberger Monoclonals, Inc). Antibodies to other cytoskeletal proteins were used as follows: anti-α-tubulin (clone DM1A, 1:1000; ICN), anti-β-tubulin (clone TUB 2.1, 1:250; ICN), anti-actin (1:500; Sigma), anti-tropomyosin (1:500; Sigma), anti-tau (1:1000; Sigma), anti-synaptophysin (clone SY38, 1:1000; Boehringer Mannheim), anti-vimentin (clone V9, 1:500; Sigma), and anti-synapsin I (1:1000; kindly provided by Drs. A. Czernik and P. Greengard, Rockefeller University). Immunolabeling was detected with ^{125}I-labeled goat anti-mouse Ig (or ^{125}I-labeled goat anti-rabbit IgG, in the case of anti-NF-66, anti-synapsin, anti-tau, anti-tropomyosin, and anti-actin antibodies) (500 cpm/μl; New England Nuclear). The resulting blots were autoradiographed with the use of intensifying screens (DuPont), and levels of immunolabeling were quantitated by densitometric scanning of autoradiograms.

Coomassie blue staining analysis of neurofilaments. For Coomassie blue staining, lanes on one-dimensional gels were loaded with 60–100 μg of protein and the gels were then stained for 2–4 hr with 0.125% Coomassie brilliant blue R (Sigma) in 50% methanol/10% acetic acid (v/v). Gels were then destained for 1 hr in 50% methanol/10% acetic acid and then for 2–3 hr in several changes of a solution containing 5% methanol/7% acetic acid. After destaining the gels, NF levels were quantitated by densitometric scanning of wet gels using a GSXL laser-enhanced densitometer (LKB). It was determined that NF levels were linear over a fivefold concentration range of brain tissue used, as measured by densitometric analysis.

Results

Chronic morphine and cocaine regulation of 200, 160, 68, and 64 kDa phosphoproteins

Figure 1 shows autoradiograms of two-dimensional gels that illustrate the effect of chronic morphine treatment on back phosphorylation in the VTA. While the overall phosphoprotein pattern in control and morphine-treated rats was very similar, a number of individual phosphoproteins were consistently altered by chronic morphine treatment. Chronic morphine significantly decreased the back-phosphorylation levels of four phosphoproteins of 200, 160, 68, and 64 kDa. These data are presented in numerical form in Table 1. It can also be seen in Figure 1 that a 58 kDa phosphoprotein known to be tyrosine hydroxylase is increased by chronic morphine, as has been reported previously (Beitner-Johnson and Nestler, 1991). In addition, chronic morphine regulated several other as yet unknown phosphoproteins of M_r 39, 51, and 71 kDa.

Interestingly, chronic cocaine was found to regulate the 200, 160, 68, and 64 kDa phosphoproteins in the same way as chronic morphine in the VTA. In Figure 2, autoradiograms of two-dimensional gels show that chronic cocaine decreased back-phosphorylation levels of these four phosphoproteins in this brain region. Numerical data are presented in Table 1. Chronic cocaine also appeared to regulate tyrosine hydroxylase as well as several other unknown phosphoproteins of 39, 51, and 71 kDa regulated by chronic morphine (see Figs. 1, 2). We have designated these various proteins as MCRPPs (morphine- and cocaine-regulated phosphoproteins).

Neither a single acute dose of morphine (10 mg/kg of morphine sulfate, s.c.) nor a single acute dose of cocaine (15 mg/kg, i.p.) with rats used 30 min later produced consistent alter-
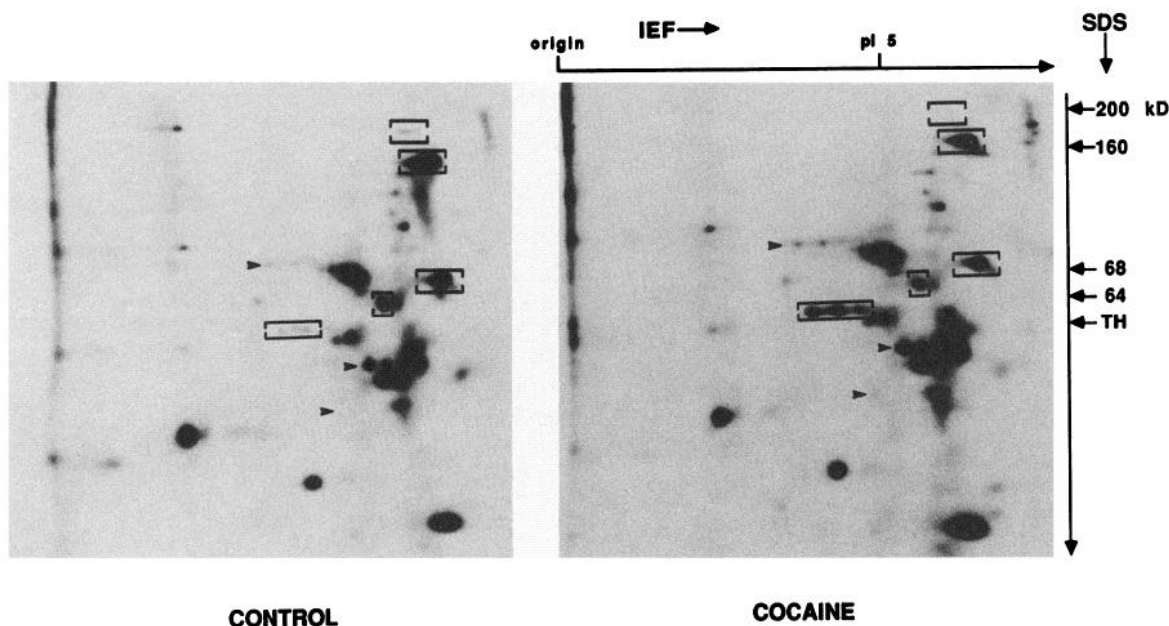
Figure 2. Autoradiograms of two-dimensional gels illustrating the effect of chronic cocaine on back phosphorylation in the VTA. Rats were treated chronically with cocaine as described in Materials and Methods. Isolated VTA nuclei were then subjected to back phosphorylation and two-dimensional gel electrophoresis, as in Figure 1. *Brackets* indicate the positions of phosphoproteins of M_r 200, 160, 68, and 64 whose back phosphorylation is decreased by both chronic cocaine and chronic morphine (see Fig. 1), and also indicate the position of a 58 kDa phosphoprotein [known to be tyrosine hydroxylase (*TH*)] whose back phosphorylation is increased by chronic cocaine and chronic morphine. Several other unknown phosphoproteins of 39, 51, and 71 kDa (indicated by *arrowheads*), which are not studied further here, also appeared to be regulated by cocaine and morphine.

ations in phosphoprotein levels in the VTA (data not shown). These findings indicate that chronic drug treatment is necessary to produce the changes reported above.

Identification of MCRPP-200, -160, -68, and -64 as NF proteins

MCRPP-200, -160, and -68 were relatively abundant proteins, as they were easily detectable by Coomassie blue staining of two-dimensional gels (Fig. 3*B*). Moreover, given the M_r of these phosphoproteins and their strongly acidic isoelectric points on two-dimensional gels, it was considered that they could represent NF proteins (see Brown et al., 1981; Tytell et al., 1981). The phosphoproteins were also found to be present at high levels in spinal cord, as measured by back phosphorylation and two-dimensional gel analysis (gels not shown; see below), which further supported the possibility that these could be NF proteins. The identity of the 200, 160, and 68 kDa phosphoproteins as NFs was confirmed by use of specific antibodies (Fig. 3*C–E*). It was found that immunolabeled NF-200 (NF-H) comigrates exactly with the 200 kDa phosphoprotein on two-dimensional gels. Likewise, the NF-160 (NF-M) and NF-68 (NF-L) immunolabeled spots comigrate with the 160 and 68 kDa phosphoproteins, respectively. In some experiments, VTA acid extracts, which had been previously labeled with ^{32}P by back phosphorylation, were ^{125}I-immunolabeled for NFs (gels not shown). This double-labeling method confirmed that the electrophoretic po-

sitions of NF-200, NF-160, and NF-68 corresponded exactly to those of MCRPP-200, -160, and -68.

MCRPP-64 was not sufficiently abundant to be visualized on Coomassie blue–stained gels. However, like the other three major NF proteins, this phosphoprotein was also present at high levels in spinal cord. The possibility was then considered that MCRPP-64 could also be an NF (or NF-related) protein. As shown in Figure 3*F*, using two-dimensional immunolabeling techniques, it was determined that this phosphoprotein corresponds to NF-66, a novel NF subunit (Chiu et al., 1989). Using the double-labeling method described above, antiserum to NF-66 (also known as α-internexin; Flienger et al., 1990) labeled a protein that comigrated exactly with MCRPP-64. By using a narrow range of ampholines in the isoelectric focusing dimension (4% ampholines, 1:10 pH 3.5–10:pH 4–6), three apparent isoforms of this protein were resolved, as shown in the inset to Figure 3*F*. Antiserum to NF-66 labeled one major immunoreactive form and two slightly more acidic minor immunoreactive forms. By double-labeling VTA acid extracts with ^{32}P and ^{125}I-secondary antibody, it was determined that the major immunoreactive isoform represents nonphosphorylated NF-66, and the two acidic isoforms correspond to ^{32}P-labeled phosphoproteins that yield phosphopeptide maps identical to those derived from MCRPP-64 on two-dimensional gels run under standard isoelectric focusing conditions (not shown). This finding corroborates a previous report of the existence of an acidic

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Figure 3. Identification of MCRPP-200, -160, -68, and -64 as NFs. *A,* Aliquots of VTA acid extracts were subjected to back phosphorylation with purified cAMP-dependent protein kinase and γ-^{32}P-ATP and to two-dimensional gel electrophoresis. Gels were then stained with Coomassie blue, destained, dried, and autoradiographed, as described in Materials and Methods. Autoradiogram of a representative gel is shown. *B,* Photograph of a Coomassie blue–stained gel, prepared as described in *A. C,* Aliquots of VTA acid extracts were subjected to back phosphorylation as described
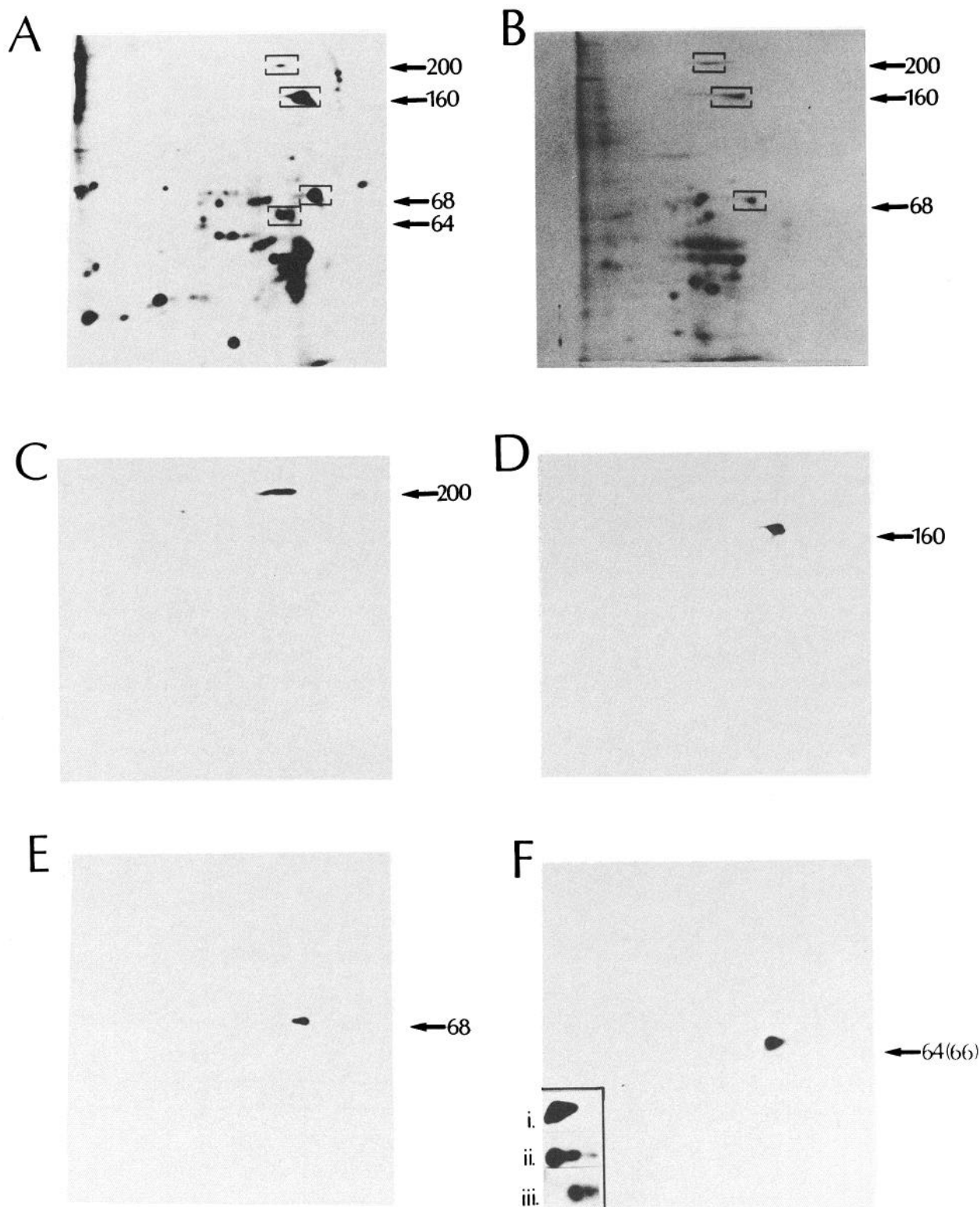
above but with non-radioactively labeled ATP and subjected to two-dimensional gel electrophoresis. Proteins were then transferred electrophoretically to nitrocellulose papers and immunolabeled for NF-200, as described in Materials and Methods. Autoradiogram of a representative two-dimensional immunoblot is shown. *D,* Aliquots of VTA acid extracts were prepared as described in *C* and immunolabeled for NF-160. *E,* Aliquots of VTA acid extracts were prepared as described in *C* and immunolabeled for NF-68. *F,* Aliquots of VTA acid extracts were prepared as described in *C* and immunolabeled for NF-66. *Inset,* Portions of autoradiograms of two-dimensional gels run under "narrow range" isoelectric focusing conditions (4% ampholines, 1:10 pH 3.5–10:pH 4–6) illustrating the effect of phosphorylation on NF-66. *i,* NF-66 immunoreactivity in nonphosphorylated VTA acid extracts. *ii,* NF-66 immunoreactivity in VTA acid extracts that were back phosphorylated with unlabeled ATP. *iii,* ^{32}P-labeled MCRPP-64 in VTA acid extracts back phosphorylated as *ii,* but in the presence of γ-^{32}P-ATP. The *brackets* in gel *A* identify the position of MCRPP-200, -160, -68, and -64. The figure shows that these phosphoproteins comigrate with Coomassie blue–stained NF proteins and immunolabeled NF-200, -160, -68, and -66.

Figure 4. Autoradiograms illustrating the effect of chronic morphine and chronic cocaine on levels of NF immunoreactivity. *A* and *B,* Rats were treated chronically with morphine (*A*) or cocaine (*B*) as described in Materials and Methods. The VTA and SN were isolated from control (−) or drug-treated (+) rats and then 25–40 µg (VTA) or 40–60 µg (SN) of crude homogenates were subjected to SDS-PAGE and immunolabeled for NF-200, NF-160, and NF-68.
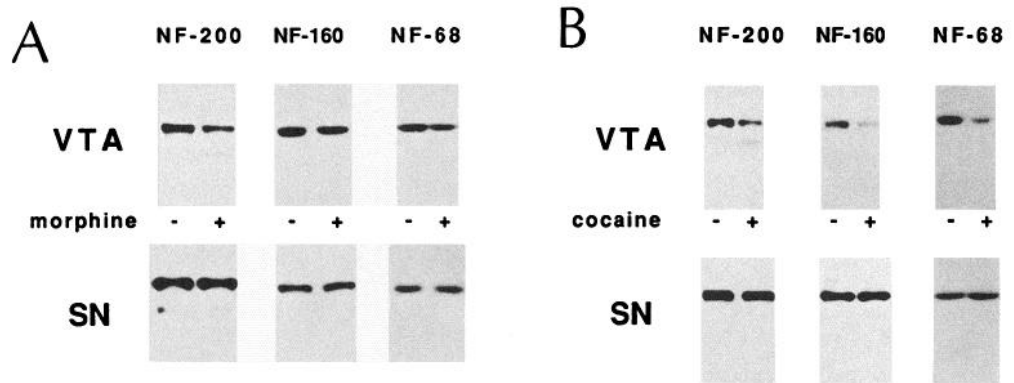
variant of this protein on two-dimensional gels (Nixon et al., 1990).

Chronic morphine and cocaine regulation of NF immunoreactivity and Coomassie blue staining

Identification of these phosphoproteins as NFs enabled us to study morphine and cocaine regulation of NFs in more detail using immunolabeling techniques. This is important because back phosphorylation could reflect changes in the phosphorylation state or total amounts of these proteins, or both (see Guitart and Nestler, 1989). Chronic morphine or chronic cocaine decreased levels of NF-200, NF-160, and NF-68 immunoreactivity in the VTA to a degree roughly comparable to the decreases measured by back phosphorylation. These findings are shown in Figure 4, with the data presented in numerical form in Table 1.

Because NFs could be readily visualized on two-dimensional Coomassie blue–stained gels, as demonstrated in Figure 3*B,* attempts were made to detect NF-200, NF-160, and NF-68 as Coomassie blue–stained bands on one-dimensional gels as has been reported previously (Brown et al., 1981; Tytell et al., 1981). The identity of the one-dimensional bands as NFs was indicated by the following observations (not shown). First, prominent phosphoproteins of 200, 160, and 68 kDa comigrated exactly with the Coomassie blue–stained proteins. Second, each of these phosphoproteins yielded one-dimensional peptide maps indistinguishable from the 200, 160, and 68 kDa bands resolved on

two-dimensional gels. Third, the phosphorylated and Coomassie blue–stained bands showed identical regional distributions; for example, both were present in the VTA and spinal cord at much higher levels than in whole cerebral cortex, consistent with what is known about the regional distribution of NF proteins (see Robinson and Anderton, 1988; Hammerschlag and Brady, 1989).

Morphine- and cocaine-induced decreases in NF-200, NF-160, and NF-68 in the VTA could be visualized on Coomassie blue–stained gels, as illustrated in Figure 5. These data are presented in numerical form in Table 1. Coomassie blue staining analysis of NFs has the advantage over immunolabeling procedures of bypassing any possible drug-induced alterations in immunogenicity due to changes in phosphorylation, as many antibodies to NFs selectively recognize specific phosphorylation states (Sternberger and Sternberger, 1983; Carden et al., 1985; Lee et al., 1987). Indeed, this may explain why the effects of chronic morphine and cocaine were generally most consistent and robust by Coomassie blue staining (see Table 1).

In addition to decreasing the total amounts of NF proteins, we next considered the possibility that chronic morphine or cocaine might also alter the phosphorylation state of the proteins. We examined this directly by using an antibody specific for highly phosphorylated states of NF-200 and NF-160 (SMI-31; Sternberger Monoclonals Inc.). VTA homogenates from rats treated chronically with morphine or cocaine were immunolabeled with this antibody. We found that, in contrast to a decrease

Table 1. Morphine and cocaine regulation of NF proteins in the VTA

	NF levels			
	NF-200[a]	NF-160	NF-68	NF-66
Morphine				
Back phosphorylation	—	71 ± 5 (12)*	76 ± 5 (11)*	83 ± 4 (12)*
Coomassie blue staining	65 ± 7 (4)*	71 ± 4 (4)*	78 ± 7 (4)*	ND
Immunolabeling	82 ± 9 (7)	67 ± 9 (12)*	86 ± 4 (10)*	100 ± 10 (10)
Cocaine				
Back phosphorylation	—	72 ± 11 (7)**	87 ± 5 (10)*	84 ± 5 (12)*
Coomassie blue staining	53 ± 6 (9)*	52 ± 5 (9)*	80 ± 3 (9)*	ND
Immunolabeling	78 ± 12 (9)	52 ± 13 (6)	69 ± 11 (9)*	73 ± 6 (7)*

Data are presented as percentage of control ± SEM; *N* is given in parentheses. ND, not detectable by Coomassie staining.
[a] Back phosphorylation of NF-200 was not quantified due to variability in the resolution of this phosphoprotein on two-dimensional gels, presumably due to its high degree of phosphorylation, as described previously by Julien and Mushynski (1982).
* $p < 0.05$ by χ^2 test.
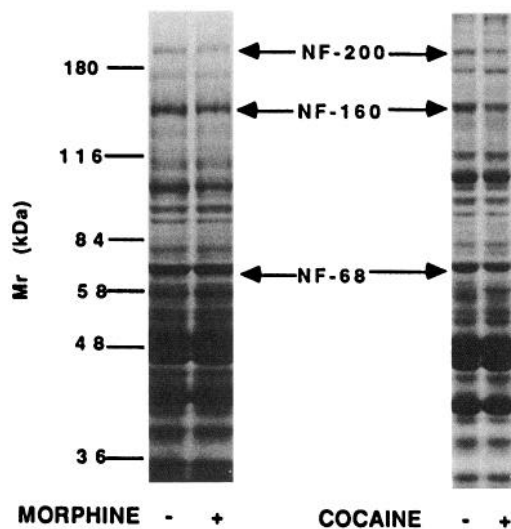** $p \approx 0.06$ by χ^2 test.

Figure 5. Photographs of Coomassie blue–stained gels illustrating the effects of chronic morphine and chronic cocaine on NF-200, NF-160, and NF-68 in the VTA. Rats were treated chronically with morphine or cocaine, as described in Materials and Methods. Total cellular extracts of VTA were isolated from control (−) and drug-treated (+) rats and subjected to SDS-PAGE and Coomassie blue staining. *Arrows* indicate the positions of NF proteins.

in Coomassie blue staining (Fig. 5) and total immunoreactivity (Fig. 4) of NFs in the VTA, levels of NF-200 and NF-160 were *not* decreased by chronic morphine or chronic cocaine treatment as measured by SMI-31 immunolabeling. In fact, there was a tendency for morphine and cocaine to *increase* SMI-31 immunolabeling (Fig. 6). This suggests that although the total amount of NFs is decreased in the VTA by chronic morphine and cocaine, the remaining NFs exist in a much more highly phosphorylated state than in the controls.

NF-66 (which was not discernable on one-dimensional gels by Coomassie blue staining) was also analyzed by immunolabeling in the VTA after chronic morphine and cocaine treatments. It was found that chronic cocaine significantly decreased NF-66 immunoreactivity in the VTA, whereas chronic morphine did not alter levels of this protein (Fig. 7, Table 1). This cocaine-induced decrease in NF-66 immunoreactivity could account for the decreased levels of the protein observed by back phosphorylation. In contrast, the morphine-induced decrease in NF-66 back phosphorylation, without a change in the total amount of the protein, could indicate that morphine decreases levels of dephospho-NF-66, that is, increases the phosphorylation state of the protein (see Guitart and Nestler, 1989). Thus, unlike NF-200, NF-160, and NF-68, NF-66 appears to be regulated differently in the VTA by chronic morphine and chronic cocaine.

Lack of effect of chronic morphine on other cytoskeletal-associated proteins in the VTA

It was of interest to determine whether chronic morphine and cocaine were selectively altering NFs in the VTA, or if other cytoskeletal or cytoskeletal-associated elements in neurons might also be disrupted by these drug treatments. To study these possibilities, levels of a number of other proteins were analyzed by immunoblotting in the VTA of rats treated chronically with morphine (Fig. 8). There were no detectable changes in levels of α- or β-tubulin, the major components of microtubules; in

tau, a microtubule-associated phosphoprotein; in actin, the major constituent of microfilaments; or in tropomyosin, a major actin-binding protein. In addition, vimentin, an intermediate-filament phosphoprotein that is structurally related to NFs but is also found in non-neural tissues, was not changed by morphine treatment. Levels of two synaptic vesicle–associated phosphoproteins, synaptophysin and synapsin I, were also unchanged in response to chronic morphine.

Regional specificity of morphine and cocaine regulation of NFs

It was important to determine whether morphine- and cocaine-induced decreases in NFs were specific to the VTA, or whether this effect was more widespread. It was found that NFs were not altered by chronic morphine in any other region examined, which included the SN (see Figure 4), the brain's other major dopaminergic nucleus; the LC, the major noradrenergic nucleus in brain; and spinal cord, which contains high levels of NFs (Table 2). In addition, NF levels did not appear to be altered by chronic morphine in whole cerebral cortex, though a quantitative analysis of NF levels in crude homogenates of cerebral cortex was not feasible, due to the much lower prevalence of NFs in this brain region (see Robinson and Anderton, 1988; Hammerschlag and Brady, 1989). To assess further the localization of morphine and cocaine regulation of NFs in the VTA,
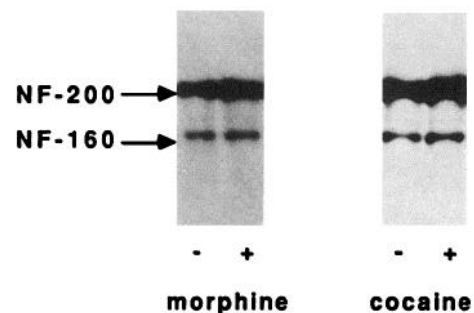

Figure 6. Autoradiograms illustrating the effect of chronic morphine and chronic cocaine on levels of highly phosphorylated NF-200 and NF-160 immunoreactivity. Rats were treated chronically with morphine or cocaine, as described in Materials and Methods. Total cellular extracts of VTA were isolated from control (−) or drug-treated (+) rats, and aliquots containing 10 μg of protein were subjected to SDS-PAGE and immunolabeled for SMI-31, as described in Materials and Methods.
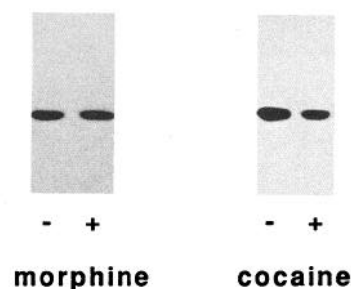

Figure 7. Autoradiograms illustrating the effect of chronic morphine and chronic cocaine on levels of NF-66 immunoreactivity. Rats were treated chronically with morphine or cocaine, as described in Materials and Methods. Total cellular extracts of VTA were isolated from control (−) and drug-treated (+) rats and subjected to SDS-PAGE and immunolabeling for NF-66, as described in Materials and Methods.

Figure 8. Lack of effect of chronic morphine on cytoskeletal or cytoskeletal-associated proteins in the VTA. Total cellular extracts of VTA, isolated from control (−) or morphine-treated (+) rats, were subjected to SDS-PAGE and immunolabeled for α- and β-tubulin, tau, actin, tropomyosin, vimentin, synaptophysin, or synapsin I.
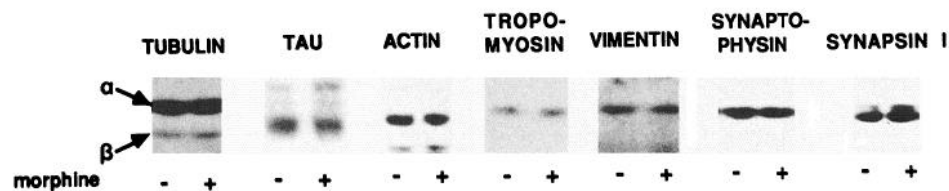
several portions of the midbrain were analyzed. The VTA and SN were removed from midbrain cross sections of control and morphine-treated rats using a 15 gauge syringe needle (i.d., 1 mm), as described in Materials and Methods. After removal of these nuclei, the area immediately surrounding the 15 gauge punch of the VTA was excised using a 12 gauge syringe needle (i.d., 2 mm), resulting in a doughnut-shaped ring of tissue. This "doughnut" and the remaining tissue in the midbrain cross section were then analyzed for NFs. Chronic morphine did not alter NFs in either the doughnut or in the remaining midbrain cross section (Table 2). In contrast to very high levels of tyrosine hydroxylase immunoreactivity within the 15 gauge VTA punch, the doughnut contained much lower levels of tyrosine hydroxylase (not shown). These data indicate that morphine regulation of NFs is localized either within the A10 dopaminergic neurons themselves, or within neuronal elements in close proximity to the dopaminergic cells of the VTA (see Discussion).

Lack of effect of other psychotropic drugs and chronic stress on NFs in the VTA

The pharmacological specificity of NF regulation in the VTA was evaluated next. Rats were treated chronically with either imipramine or haloperidol, two psychotropic drugs without reinforcing properties. Neither imipramine, an antidepressant drug, nor haloperidol, an antipsychotic drug, altered NFs in the VTA after chronic treatment as measured by immunoblot analysis. The possibility that NF regulation could be stress related was also considered. However, chronic cold stress, a procedure known to induce a number of biochemical alterations in the brain noradrenergic system (Richard et al., 1988; Melia et al., 1992), did not produce any significant changes in NF levels in the VTA. These data are summarized in Figure 9. In addition, chronic implantation of placebo morphine pellets produced no detectable changes in NF-200, NF-160, NF-68, or NF-66 as measured by direct back phosphorylation (data not shown), providing further evidence against a stress-related effect.

Discussion

This study examines the involvement of NFs in the chronic actions of morphine and cocaine in the VTA, a brain region that plays a critical role in mediating the rewarding properties of opiates, cocaine, and other drugs of abuse. We show that chronic morphine and chronic cocaine share the common action of decreasing levels of NF-200, NF-160, and NF-68 in this dopaminergic brain reward region, an effect not seen after acute morphine or cocaine administration. NFs were not altered in response to chronic treatment with imipramine or haloperidol, two psychotropic drugs without reinforcing properties, or by chronic cold or surgical stress. In contrast to NFs, levels of α- and β-tubulin, tau, actin, vimentin, synaptophysin, and synapsin I were not altered by chronic morphine treatment in the VTA, suggesting that morphine regulation of NFs does not reflect a general disruption of the neuronal cytoskeleton within this brain region. The results are consistent with the possibility that a specific downregulation of NFs may represent part of a common molecular basis for morphine and cocaine addiction mediated by the VTA.

In these studies, we have assayed NFs in three ways: by back phosphorylation, immunolabeling, and Coomassie blue staining. Taken together, the three measurements indicate that chronic morphine and chronic cocaine decrease the total amounts of NF-200, NF-160, and NF-68 in the VTA. We also found that chronic morphine and cocaine produced an apparent increase in the phosphorylation state of NF-200 and NF-160, by using SMI-31, a monoclonal antibody specific to the highly phosphorylated forms of these proteins. It is not known whether NF-68 is also increased in phosphorylation state, as phosphorylation-specific antibodies to this protein are presently unavailable, presumably due to the much lesser degree of phosphorylation of this protein (1–3 mol PO_4/mol protein) compared to NF-160 (8–26 mol PO_4/mol protein) and NF-200 (13-104 mol PO_4/mol protein) (Shaw, 1991).

Table 2. Regional specificity of chronic morphine regulation of NF proteins

| | NF immunoreactivity | | |
	NF-200	NF-160	NF-68
VTA	82 ± 9 (7)	67 ± 9 (12)*	86 ± 4 (10)*
SN	100 ± 16 (10)	109 ± 11 (12)	102 ± 6 (14)
LC	100 ± 7 (10)	102 ± 9 (9)	110 ± 9 (9)
Midbrain[a]	101 ± 21 (5)	96 ± 20 (5)	99 ± 9 (6)
VTA "doughnuts"[b]	103 ± 17 (6)	106 ± 14 (6)	100 ± 10 (6)
Spinal cord	104 ± 8 (6)	103 ± 7 (6)	97 ± 3 (6)

Data are presented as percentage of control ± SEM; N is given in parentheses.

[a] Midbrain: coronal sections of midbrain at the level of the VTA from which VTA and SN nuclei have been excised.

[b] VTA "doughnuts": 12 gauge punches of tissue surrounding 15 gauge punches of VTA nuclei.
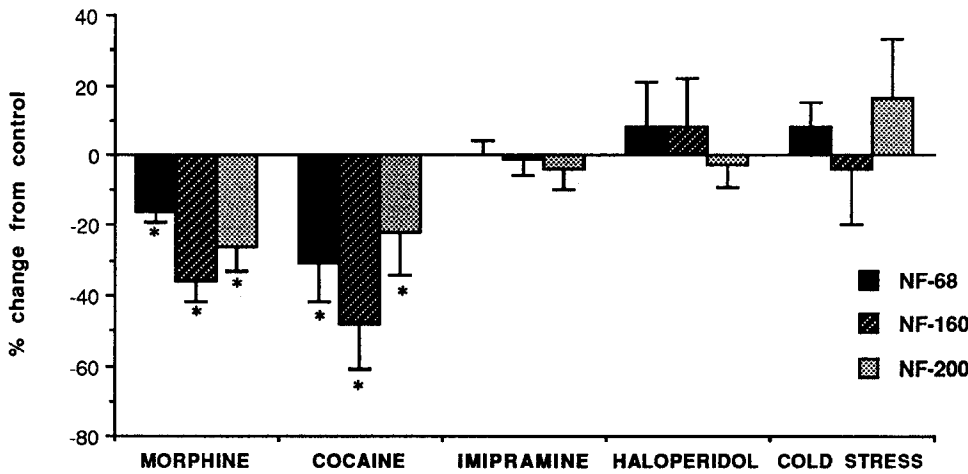
* $p < 0.05$ by χ^2 test.

Figure 9. Pharmacological specificity of morphine and cocaine regulation of NFs in the VTA. Rats were treated chronically with either morphine, cocaine, imipramine, or haloperidol or were subjected to 5 d of cold stress, as described in Materials and Methods. Total cellular extracts of VTA were isolated from control and treated rats and subjected to SDS-PAGE. Levels of NF-200, NF-160, and NF-68 were determined by immunolabeling or Coomassie blue staining, as described in Materials and Methods. Data are expressed as percentage difference from control and represent the mean ± SEM from 5–15 rats in each treatment group. *, $p < 0.05$ by χ^2 test.

It is uncertain what impact such alterations in NFs would have on the functional properties of the VTA. NFs are known to be associated with axonal transport (Hoffman and Lasek, 1975; Tytell et al., 1981; Hammerschlag and Brady, 1989), although the precise role subserved by NF proteins remains unknown. A variety of *in vivo* manipulations, including axotomy, aluminum intoxication, and chronic β,β'-iminodipropionitrile intoxication are all known to lead to decreased NF levels and to decreased rates of axonal transport (Hoffman et al., 1984; Tronosco et al., 1985; Goldstein et al., 1987; Watson et al., 1989). Furthermore, increased phosphorylation of NFs is also correlated with decreased rates of axonal transport. There is a progressive *increase* in the degree of phosphorylation of NFs as they are transported from the cell body along the axon (Sternberger and Sternberger, 1983; Hoffman et al., 1985; Carden et al., 1987; Nixon et al., 1987; Pestronk et al., 1990), which has been proposed to be the mechanism by which NF velocity becomes progressively decreased as NF proteins are concomitantly transported distally (Carden et al., 1987; Lee et al., 1987). In addition, *in vivo* axotomy (Goldstein et al., 1987) and chronic β,β'-iminodipropionitrile intoxication (Watson et al., 1989) also lead to increased phosphorylation of NFs, in concert with decreasing total levels of these proteins. Finally, NFs from rats made experimentally diabetic exist in an increased state of phosphorylation (Pekiner and McLean, 1991), which also correlates with the decreased axonal transport rates observed in diabetic animals (Macioce et al., 1989).

Given the above evidence, our findings that NFs are (1) decreased in total amount and (2) present in an increased state of phosphorylation in the VTA after chronic morphine and cocaine treatments imply conditions of decreased axonal transport within this brain region. Indeed, we have recently found that chronic morphine does impair axonal transport in the mesolimbic dopamine system (Beitner-Johnson and Nestler, 1992). This finding may be related to our previous demonstration, that although chronic morphine and cocaine increase levels of tyrosine hydroxylase immunoreactivity in VTA cell bodies, there is *not* a corresponding increase in tyrosine hydroxylase immunoreactivity in the dopaminergic nerve terminals in the nucleus accumbens (Beitner-Johnson and Nestler, 1991). Because tyrosine hydroxylase in the NAc is localized virtually entirely in nerve terminals derived from the VTA (Swanson, 1982), the lack of correspondingly elevated enzyme levels in NAc terminals could be explained by slower or less effective transport of the enzyme

along these dopaminergic axons. Taken together, our findings suggest the possibility that chronic use of morphine or cocaine may alter the structural features of mesolimbic dopamine neurons in such a way as to reduce the ability of these cells to transmit dopaminergic signals to neuronal elements in the NAc. According to this view, long-term opiate or stimulant use might impair the brain's endogenous reward system, which could have a major impact on motivation and drug-seeking behavior. Our similar findings with Lewis and Fischer 344 rats (see below) suggest that populations that are genetically predisposed to drug addiction may have a similar type of mesolimbic dopamine impairment inherently. Although much further work is needed to study the validity of these and many other interpretations, the findings indicate that alterations in NFs reflect (and possibly underlie) a common functional effect of chronic morphine and chronic cocaine in the VTA (for further discussion, see Beitner-Johnson et al., 1992).

In contrast to NF-200, NF-160, and NF-68, regulation of NF-66 was different in response to chronic morphine and cocaine treatments. While chronic cocaine decreased the total amount of NF-66, chronic morphine induced an apparent increase in phosphorylation state of the protein without a change in its total amount. This may be related to some of the distinct actions of morphine and cocaine in the VTA. Systemic morphine and cocaine have opposite acute electrophysiological actions on VTA neurons (excitatory vs. inhibitory) (Matthews and German, 1984; Einhorn et al., 1988), as well as markedly different acute behavioral effects (sedative vs. stimulant). On the other hand, a morphine-induced increase in phosphorylation state of NF-66 could be functionally equivalent to a decrease in the total amount of this protein, based on the studies of NF-160 and NF-200 phosphorylation and axonal transport, as described above. However, as of yet, little is known about the function of NF-66, other than that it is a neural-specific protein that shares homology with the three major NFs and can assemble into 10 nm (i.e., intermediate) filaments *in vitro* (Chiu et al., 1989; Flienger et al., 1990).

NFs were studied here by back phosphorylation with purified cAMP-dependent protein kinase. The three major NF proteins, NF-200, NF-160, and NF-68, have been reported to be phosphorylated both *in vivo* and *in vitro* by multiple protein kinases, including cAMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase, protein kinase C, and a cAMP/calcium-independent NF-associated protein kinase (Leterrier et

al., 1981; Vallano et al., 1985; Pant et al., 1986; Sihag et al., 1988). The protein kinase(s) responsible for morphine and cocaine regulation of NF phosphorylation *in vivo* remains unknown. The phosphorylation of NF-66 has not yet been characterized. We have found that NF-66 is phosphorylated by an endogenous calcium/calmodulin-dependent protein kinase activity in subcellular fractions of brain, but is not an endogenous substrate for cAMP-dependent protein kinase (D. Beitner-Johnson, X. Guitart, and E. J. Nestler, unpublished observations). This probably explains why the majority of NF-66 remains in a nonphosphorylated state even after back phosphorylation with purified cAMP-dependent protein kinase (see Fig. 3*F*). There is a preliminary report of the phosphorylation of NF-66 by several protein kinases *in vitro* (Nixon et al., 1990); however, the protein kinase that regulates the phosphorylation of this protein *in vivo* remains unknown.

One important issue raised by this study, as alluded to above, is the cellular localization of the morphine- and cocaine-induced decreases in NF levels. Several pieces of evidence suggest that drug regulation of NFs may occur within dopaminergic neurons themselves. First, drug regulation of NFs was observed only in the VTA, a brain region enriched in dopaminergic neurons known to play a critical role in both opiate and cocaine reward (Roberts and Koob, 1982; Bozarth and Wise, 1984; Bozarth, 1986). Second, chronic morphine and cocaine increase levels of tyrosine hydroxylase specifically in the VTA (Beitner-Johnson and Nestler, 1991). This demonstrates that the drugs do indeed produce some alterations in VTA dopaminergic neurons. Third, we also demonstrated previously that levels of tyrosine hydroxylase in the VTA are higher in Lewis rats versus Fischer rats (Beitner-Johnson et al., 1991), and that levels of NFs in the VTA are lower in Lewis versus Fischer rats (Guitart et al., 1992). This further establishes some association between higher levels of tyrosine hydroxylase and lower levels of NFs in the VTA, and could suggest the involvement of the same cell type in both phenomena. Of course, the VTA is a heterogeneous region (Phillipson, 1979; Poirier et al., 1983), and it is possible that drug regulation of NFs may be occurring in other neuronal elements located in this area. However, the fact that no change in NFs was observed in the "doughnut" of tissue directly surrounding the VTA indicates that if morphine and cocaine regulation of NFs does not occur within VTA dopaminergic neurons, then such drug regulation would be occurring either in neuronal cell types or in specific populations of axonal processes particular to this region. In any event, morphine and cocaine regulation of NFs in these non-dopaminergic elements, or in the dopaminergic neurons themselves, would be expected to have important implications for the functional effects of these drugs on the VTA.

The findings of this study establish that NF proteins represent a previously unknown target of chronic opiate and chronic cocaine action. Although the functional consequences of decreased NFs remain to be established, the results raise the exciting possibility of morphine- and cocaine-induced changes in VTA structure and function. As cited above, we have found differences in basal levels of NFs and tyrosine hydroxylase in Lewis versus Fischer rats (Beitner-Johnson et al., 1991; Guitart et al., 1992), two genetically inbred strains that exhibit different preferences for opiates, cocaine, and alcohol (Li and Lumeng, 1984; Suzuki et al., 1988; George and Goldberg, 1989; Terwilliger et al., 1991b). Together, these findings support the hypothesis that regulation of NFs and tyrosine hydroxylase could

represent common mechanisms by which these drugs alter mesolimbic dopamine function and thereby produce aspects of drug addiction.

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