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Influence of Cocaine on the JAK–STAT Pathway in the Mesolimbic Dopamine System

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Chronic exposure to cocaine produces characteristic biochemical adaptations within the rat ventral tegmental area (VTA), a brain region rich in dopaminergic neurons implicated in the reinforcing and locomotor-activating properties of cocaine. Some of these changes are mimicked by chronic ciliary neurotrophic factor (CNTF) infusions into the same brain area. We show in this study that chronic cocaine treatment regulates the signal transduction pathway used by CNTF specifically in the VTA. There is an increase in immunoreactivity of Janus kinase (JAK2), a CNTF-regulated protein tyrosine kinase, in the VTA after chronic but not acute cocaine administration. This increase is not seen in the nearby substantia nigra or several other brain regions studied. Furthermore, this increase in JAK2 is not seen after chronic administration of other psychotropic drugs and was not observed for JAK1. The increase in JAK2 levels is associated with an increased responsiveness of the system to acute CNTF infusion into the VTA, as measured by induction in this brain region of signal transducers and activators of transcription (STAT) DNA binding activity and of Fos-like proteins, two known functional endpoints of JAK activation. Double-labeling immunohistochemical studies show that JAK2 immunoreactivity in the VTA is enriched in dopaminergic and nondopaminergic cells, both of which exhibit increased JAK2 immunoreactivity after chronic cocaine treatment. These findings suggest a scheme whereby some of the effects of chronic cocaine on VTA dopaminergic neurons are mediated directly by regulation of the JAK–STAT pathway in these cells, as well as perhaps indirectly by regulation of this pathway in nondopaminergic cells.

Key words: JAK; STAT; c-Fos; tyrosine hydroxylase; VTA; glia

The mesolimbic dopamine system is implicated in the reinforcing effects of cocaine and other drugs of abuse (Bozarth and Wise, 1986; Kuhar et al., 1991; Koob, 1992). This brain pathway consists of dopaminergic neurons in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAc) and other forebrain structures. Several molecular adaptations have been observed within the VTA and NAc after chronic drug exposure (Nestler, 1992; Nestler et al., 1993; Striplin and Kalivas, 1993; Cerutti et al., 1994; Self and Nestler, 1995). One of the most consistent adaptations is an increase in levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, in the VTA after chronic cocaine, opiate, amphetamine, and ethanol exposure (Beitner-Johnson and Nestler, 1991; Hurd and Herkenhan, 1992; Sorg et al., 1993; Vrana et al., 1993; Ortiz et al., 1995). There is also evidence for drug regulation of glial cells in the VTA, namely, alterations in glial fibrillary acidic protein (GFAP) (Beitner-Johnson et al., 1993; Ortiz et al., 1995).

Previous research has demonstrated an interaction between neurotrophic factors and drugs of abuse at the level of the VTA. Chronic infusion of brain-derived neurotrophic factor (BDNF) (2.5 μg/d) into the VTA both prevents and reverses the ability of cocaine or morphine to increase levels of TH in this brain region (Berhow et al., 1995). In contrast, chronic intra-VTA infusion of ciliary neurotrophic factor (CNTF) (1.5 μg/d) alone produced a significant increase in TH immunoreactivity, with no further increase seen when cocaine or morphine is also given. Similar effects were observed for GFAP (Berhow et al., 1995). The finding that CNTF can mimic some of the long-term effects of cocaine and opiates in the VTA in a nonadditive manner raises the possibility that some of the effects of the drugs could conceivably be mediated via perturbation of the signal transduction pathway used by CNTF.

CNTF belongs to a family of cytokines, which also includes oncostatin M, leukemia inhibitory factor, interleukin 6, and granulocyte colony stimulating factor. CNTF was identified originally as a trophic factor for ciliary ganglion neurons, but was subsequently shown to promote the survival of motor, hippocampal, and monoaminergic neurons (Ip et al., 1991; Ip and Yancopoulos, 1996). The ways in which the other cytokines regulate neural function remain less well characterized.

The effects of CNTF and related cytokines are mediated via the GP130 receptor family. Specificity arises from the α component of the receptor, which is unique to the given ligand. Cytokine binding to the α component triggers its association with two transmembrane β components. Associated with the β components are cytoplasmic protein tyrosine kinases belonging to the Janus kinase family (JAK1, JAK2). These enzymes are phosphorylated and activated when the tripartite receptor complex is formed after ligand binding. Phosphorylated JAK, in turn, allows for the rec-

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ognition of the activated receptor complex by a family of transcription factors known as signal transducers and activators of transcription (STAT). STAT1 and STAT3 are the STAT family members thought to be regulated by CNTF. Phosphorylated STATs dimerize and translocate to the nucleus, where they bind to specific DNA regulatory elements and regulate gene expression (Stahl et al., 1990; Darnell et al., 1994; Symes et al., 1994; Guschin et al., 1995). CNTF and its receptor complex have been localized to both neurons and glia within the CNS (Squinto et al., 1990; Stockli et al., 1991; Carroll et al., 1993; Symes et al., 1993; Henderson et al., 1994). Similarly, CNTF elicits functional effects in both neurons (e.g., regulation of TH) and glia (e.g., regulation of GFAP) *in vivo* and in mixed populations of cells *in vitro* (Ip et al., 1992; Hagg et al., 1993; Louis et al., 1993b; Burnham et al., 1994; Lewis et al., 1994; Berhow et al., 1995; Winter et al., 1995; Ip and Yancopoulos, 1996).

The objective of the present study was to investigate potential cross-talk between the JAK–STAT pathway and chronic exposure to drugs of abuse. We show here that chronic administration of cocaine, but not of several other psychotropic drugs, upregulates JAK2 expression specifically in the VTA. Our results raise the novel possibility that cocaine-induced adaptations in the JAK–STAT pathway could contribute to some of the long-term effects of cocaine on mesolimbic dopamine function.

MATERIALS AND METHODS

In vivo *neurotrophic factor infusions and drug treatments.* Male Sprague Dawley rats (initial weights 260–275 gm; CAMM, Wayne, NJ) were used in these studies. Neurotrophin and cytokine infusions involved implantation of osmotic minipumps (Alzet Model 2002) that provide a constant infusion of 0.5 ml/hr for 14 d. CNTF and BDNF human recombinant growth factors, expressed in *Escherichia coli*, were provided by Regeneron Pharmaceuticals (Tarrytown, NY). CNTF and BDNF were delivered, as described previously (Berhow et al., 1995), in a solution containing 10 mM sodium phosphate, pH 7.4, 0.9% NaCl, and 1% bovine serum albumin. The doses of CNTF (1.5 μg/d) and BDNF (2.5 μg/d) were based on previous research (Berhow et al., 1995). Animals were anesthetized with 3 mg/kg of Equithesin and implanted with an osmotic minipump connector cannula (28 gauge, 22 gauge connector; Plastic Products Company). Midline VTA coordinates of −5.3 mm anterior-posterior (AP) and 8.4 mm dorsal-ventral (DV) were used. Osmotic pumps were placed subcutaneously between the scapulae and connected to the cannula via PE60 tubing cut to 2.5 cm in length. Each end was sealed with LocTite glue. The cannula was secured in place with dental cement. Control rats were implanted with osmotic pumps containing a vehicle solution. The effective delivery of CNTF via the minipumps was monitored as before by weight loss and increased levels of TH in the VTA (Berhow et al., 1995).

Acute cytokine administration involved techniques similar to those described above. The tip of a Hamilton syringe needle (25 gauge) containing vehicle or CNTF solution was lowered to −8.4 mm DV, at −5.3 mm AP. The doses (0.08, 0.1, or 0.5 μg) were delivered in 1 μl over a 2 min period. The syringe needle then remained inserted for an additional 5 min before removal. Animals were killed 90 min (STAT binding) or 3 hr (c-Fos) later. The effective delivery of CNTF was demonstrated by dose-dependent regulation of these two endpoints.

Three different cocaine treatments were used: chronic, chronic–acute, and acute. Chronic cocaine treatment involved administration of cocaine twice daily via intraperitoneal injections of cocaine-HCl (15 mg/kg; National Institute on Drug Abuse) in 0.9% NaCl for 10 d; brains were removed from decapitated rats 1 d after the last cocaine injection (Berhow et al., 1995). Chronic–acute treatment was identical to chronic treatment except that the brains were removed 1 hr after the last cocaine injection. Acute cocaine treatments involved a single dose of cocaine-HCl (20 mg/kg) administered via intraperitoneal injections; the brains were removed 1 hr later. Control rats received equivalent saline injections.

Chronic morphine treatment involved implantation of one morphine pellet (containing 75 mg of morphine base; National Institutes on Drug Abuse) subcutaneously daily for 5 d while rats were under light halothane anesthesia. Control rats received sham surgery. Animals were used on day 6 (Berhow et al., 1995). Chronic desipramine and fluvoxamine treatments involved once daily intraperitoneal injections of 15 mg/kg and 10 mg/kg, respectively, as described (Nibuya et al., 1995). Both drugs were dissolved in saline.

Immunolabeling of proteins: JAK2, JAK1, and c-Fos. Brains were removed from decapitated rats and cooled in ice-cold physiological buffer. The VTA, substantia nigra, NAc, and caudate-putamen were obtained as 12–15 gauge punches from coronal cross-sections of brain, as described previously (Beitner-Johnson et al., 1993). The remaining brain regions and cervical sections of spinal cord were obtained via gross dissection. Brain samples (for JAK2 and JAK1) were homogenized in 10–15 mg/ml of 1% SDS and adjusted to contain final concentrations of 50 mM Tris, pH 6.7, 4% glycerol, 2% SDS, and 2% 2-mercaptoethanol, with bromophenol blue as a marker. Samples for c-Fos blots were homogenized in a different buffer, exactly as described (Hope et al., 1994). Samples were then boiled for 2 min, and aliquots containing 20 μg (JAK2, JAK1) or 50 μg (c-Fos) of total protein were subjected to SDS-PAGE with 6% acrylamide and 0.3% bisacrylamide in the resolving gels. Proteins were transferred electrophoretically to nitrocellulose papers, which were blocked with 2% nonfat dry milk in buffer containing 10 mM sodium phosphate, pH 7.2, 140 mM NaCl, and 0.05% Tween 20 (Sigma, St. Louis, MO). Proteins were then immunolabeled with the following antibodies: anti-JAK2 and anti-JAK1 (diluted 1:2000; UBI) and anti-Fos-related antigen (diluted 1:4000; M. Iadarola, National Institutes of Health) (Young et al., 1991). Primary antibodies were detected with peroxidase-linked secondary antibodies from Vector (Burlingame, CA) and detected with enhanced chemiluminescence (Amersham, Arlington Heights, IL) and autoradiography. The resulting autoradiograms were quantified via a Macintosh-based image analysis system with National Institutes of Health image software. Under the immunoblotting conditions used, levels of the various proteins labeled were linear over a threefold range of VTA extract concentration. Resulting blots were stained with amido black to confirm equal loading of the samples. Specificity of the anti-JAK2 and anti-JAK1 antibodies was established by the observation that the major immunoreactive band recognized by each antibody corresponded to the published M_r of JAK2 or JAK1, respectively, and was specifically obliterated by preabsorbing the antibody with the peptide antigen (JAK2: DSQRKLQFYEDKHQLPAPKC; JAK1: KTLIEKERFYSRCRPVTPSC).

Gel mobility shift assays. Whole-cell extracts were made from frozen, bilaterally dissected VTA or substantia nigra by homogenizing the tissue in 25 ml of the following buffer: 20 mM HEPES, pH 7.9, 12.5% glycerol, 0.4 M NaCl, 0.06% NP40, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium vanadate, 0.4 mM microcysteine, and 5 μg/ml each of aprotonin and leupeptin. After homogenization the tissue was incubated at 4°C for 30 min, and the supernatant was collected after centrifugation at 16,000 × *g* for 5 min at 4°C.

A double-stranded oligonucleotide referred to as mSIE (5′-CAGTTCCGTCAATC) (Wagner et al., 1990) was labeled using Superscript Reverse Transcriptase (Life Technologies, Gaithersburg, MD) and [α-^{32}P] dCTP (3000 Ci/mmol). For the binding reaction, 4 μl of whole-cell extract was incubated for 20 min at 4°C with 1 ng of ^{32}P-labeled mSIE probe in a final volume of 20 ml with a buffer containing 8 mM HEPES, pH 7.9, 20 mM KPO_4, 8% glycerol, 0.3 mM EDTA, 1.2 mM DTT, 0.2 mM PMSF, and 0.5 μg dIdC. Protein–DNA complexes were then separated in 6% acrylamide gels by PAGE and visualized with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). This procedure yields three tightly spaced bands, which represent specific STAT binding based on supershift assays with specific anti-STAT antibodies. In these experiments, 1 μl of either a specific anti-STAT3 antibody (Santa Cruz Biotechnology, Tebu, France) or a specific anti-STAT1 antibody (Transduction Labs, Lexington, KY) was mixed with the binding reaction for an additional 30 min before the addition of radiolabled probe (Symes et al., 1994). These assays identified the uppermost band as STAT3:3 homodimers, the lowermost band as STAT1:1 homodimers, and the middle band as STAT3:1 heterodimers.

Immunohistochemical analysis. For immunohistochemical studies, saline- and cocaine-treated rats were anesthetized with pentobarbital and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were post-fixed in 4% paraformaldehyde for 2 hr, cryoprotected in 20% glycerol overnight, and cut coronally on a sliding microtome at 20 μm. Sections were stored in 0.1% sodium azide in 0.1 M phosphate buffer.

Sections were pretreated for 10 min with 3% H_2O_2, for 30 min with 5% normal goat serum, and overnight at room temperature with a mixture of rabbit polyclonal JAK2 antiserum (1:15,000; UBI) and mouse monoclonal TH (1:800; Chemicon, Temecula, CA) or a mixture of polyclonal

JAK2 antiserum and monoclonal GFAP (1:10,000; Sigma). Sections were then incubated for 2 hr with a mixture of Texas Red-conjugated goat anti-rabbit IgG (1:500; Jackson Immunochemical Research, West Grove, PA) and fluorescein-conjugated goat anti-mouse IgG (1:10,000; Jackson Immunochemical Research). After sections were mounted, they were dried and coverslipped with 10% 0.1 M PO_4 buffer containing 20% glycerol and 5 mg/ml 1,4-diazabicyclo[2, 2, 2]-cotane (Aldrich, Milwaukee, WI). Sections then were examined with a confocal microscope (Bio-Rad MRC 600; Bio-Rad, Richmond, CA).

We performed three sets of controls to confirm the specificity of double-immunostaining. First, preabsorption of JAK2 antiserum with the peptide antigen blocked the observed staining. This finding indicates that this polyclonal antiserum does not contain antibodies that recognize other antigens. Specificity of the monoclonal TH and GFAP antibodies has been well characterized previously (Beitner-Johnson and Nestler, 1991; Beitner-Johnson et al., 1993). Second, omission of the primary antiserum resulted in no staining. This finding indicates that the secondary IgGs lack nonspecific binding to tissue sections under the experimental conditions used. Third, omission of one primary antibody in the double-labeling experiment eliminated staining of the omitted antibody without affecting the other staining. This finding indicates that the secondary antibodies did not show significant cross-reactivity and that labeling with one antibody did not affect labeling of the other.

RESULTS

Regional distribution of JAK1 and JAK2 in rat brain

An examination of the regional distribution in the brain of JAK1 and JAK2 immunoreactivity, as measured by immunoblotting, was performed as an initial step in our study (Fig. 1). Both forms of JAK displayed a widespread distribution in the brain, with similar relative levels seen in most brain regions. The VTA, however, contained a higher level of JAK2 relative to other regions compared with JAK1, although a direct comparison of the absolute levels of the two enzymes was not possible. The relative distribution observed for JAK1 and JAK2 immunoreactivity correlated well with the distribution of the CNTF receptor α component as described previously (Squinto et al., 1990; MacLennan et al., 1996).

Effect of cocaine on JAK2 immunoreactivity: regional and pharmacological specificity

We next examined the effect of chronic cocaine treatment on JAK levels in the VTA using immunoblotting. As shown in Figure 2, levels of JAK2 immunoreactivity were increased by 48% in the VTA after chronic cocaine exposure. There was no difference between cocaine- and saline-treated rats with respect to JAK2 immunoreactivity in the substantia nigra, a region anatomically related to the VTA, or in the NAc and frontal cortex, two VTA projection regions. In contrast to the increase in JAK2 immunoreactivity seen within the VTA after chronic cocaine exposure, there was no drug-induced change in levels of JAK1 immunoreactivity in this brain region (Fig. 2*A*).

In the chronic cocaine treatment paradigm, rats were killed 1 d after the last cocaine injection. Given the half-life of cocaine of < 30 min, changes observed in JAK2 levels potentially could be attributed to either chronic cocaine exposure or cocaine withdrawal. To study these possibilities, both acute and chronic–acute paradigms were used. In the chronic–acute paradigm, animals were killed 1 hr after the last cocaine injection. As with the chronic cocaine paradigm, a significant increase in levels of JAK2 immunoreactivity was observed in the VTA in the chronic–acute paradigm (Table 1). In contrast, a single acute injection of cocaine did not produce any change in levels of JAK2 immunoreactivity in this brain region (Table 1).

Although cocaine can effectively inhibit all known monoamine reuptake transporters, most of its actions on the mesolimbic dopamine system have been attributed to its inhibition of dopamine reuptake (see introductory remarks). To study the pharmacological specificity of the JAK2 increase in the VTA seen with chronic cocaine treatment, other chronic drug treatment paradigms were used, including desipramine (a specific norepinephrine reuptake inhibitor) and fluvoxamine (a specific serotonin reuptake inhibitor). Chronic exposure to these drugs failed to alter levels of JAK2 immunoreactivity in the VTA (Table 1). Chronic administration of morphine also failed to alter JAK2 levels in this brain region (Table 1).

Given the increase in JAK2 levels after prolonged perturbation of the system by chronic cocaine, we tested whether a similar adaptation of this CNTF-regulated kinase would be observed after chronic intra-VTA infusions of CNTF itself. After a 10 d

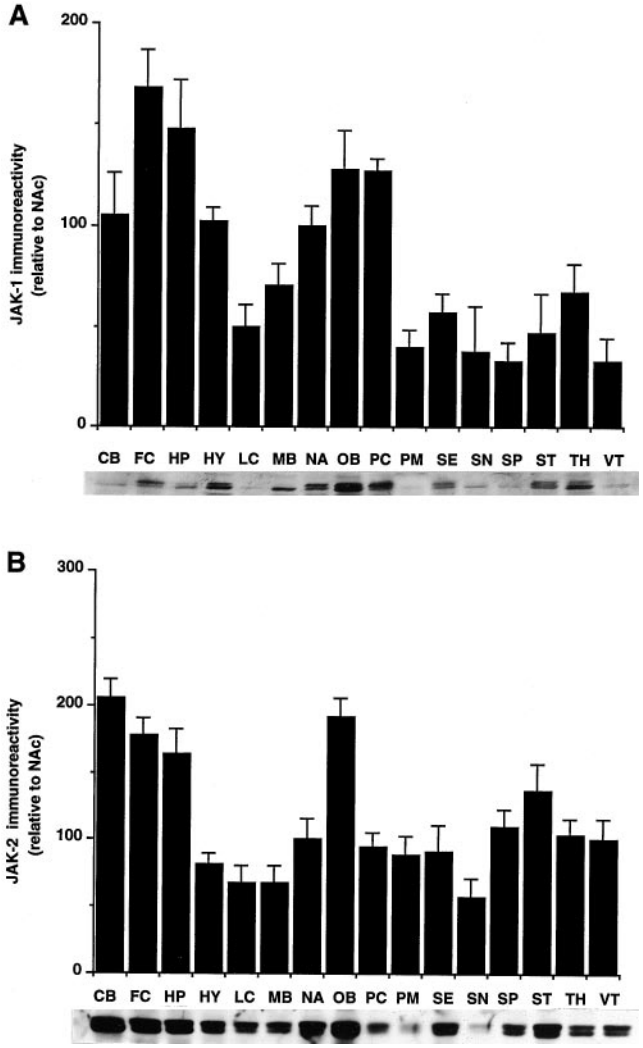

Figure 1. Regional distribution of (*A*) JAK1 and (*B*) JAK2 immunoreactivity in rat brain. Aliquots (20 μg of protein) of SDS-solubilized extracts were subjected to SDS-PAGE, and resulting gels were processed for blot immunolabeling of JAK1 or JAK2 using anti-JAK1 or anti-JAK2 antibodies as described in Materials and Methods. The *top panels* of *A* and *B* summarize the data (mean ± SEM) obtained from four animals. All data are expressed as percentage of immunoreactivity relative to nucleus accumbens (*NAc*) normalized to 100%. *CB*, Cerebellum; *FC*, frontal cortex; *HP*, hippocampus; *HY*, hypothalamus; *LC*, locus coeruleus; *MB*, midbrain; *NA*, nucleus accumbens; *OB*, olfactory bulb; *PC*, parietal cortex; *PM*, pons medulla; *SE*, septum; *SN*, substantia nigra; *SP*, spinal cord; *ST*, striatum; *TH*, thalamus; *VT,* ventral tegmental area. Each panel shows portions of resulting immunoblots obtained from a representative rat.

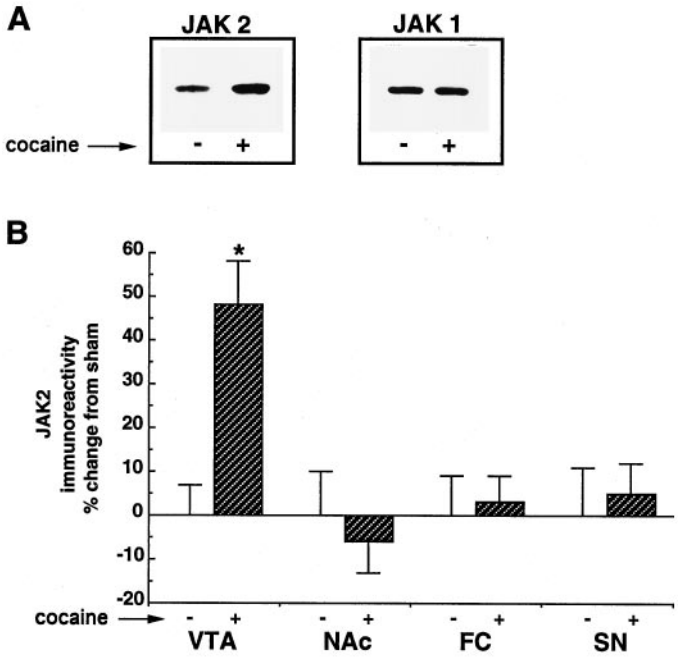

Figure 2. Regulation of JAK2 immunoreactivity by chronic cocaine. *A*, Representative autoradiograms of VTA samples illustrating the chronic cocaine-induced increase in JAK2 immunoreactivity (*left*), with no change in JAK1 immunoreactivity (*right*). *B*, Graph of JAK2 immunoreactivity, expressed as percentage change from sham, with and without chronic cocaine treatment in four brain regions: *VTA*, nucleus accumbens (*NAc*), frontal cortex (*FC*), and substantia nigra (*SN*). Data are expressed as mean ± SEM (*$p < 0.05$ vs sham by χ^2 test). The brain regions and numbers of animals used without and with cocaine, respectively, are as follows: VTA (12, 12), NAc (8, 8), FC (8, 8), and SN (8, 8).

infusion of CNTF (1.5 μg/d) into the VTA, levels of JAK2 immunoreactivity were increased by 35% relative to vehicle infusions (Table 1). In contrast, no change in JAK2 immunoreactivity was observed 90 min or 2 hr after a single acute infusion of CNTF (0.5 μg) into this brain region (not shown). In addition, no change in JAK2 levels was seen in animals receiving chronic intra-VTA infusion of BDNF.

Table 1. Pharmacological analysis of the regulation of JAK2 immunoreactivity in the VTA

Control	100 ± 12
Chronic cocaine	148 ± 11*
Chronic–acute cocaine	145 ± 14*
Acute cocaine	96 ± 6
Chronic morphine	105 ± 8
Chronic desipramine	87 ± 16
Chronic fluvoxamine	88 ± 17
Chronic CNTF	136 ± 17*
Chronic BDNF	93 ± 9

Treatment paradigms included chronic, chronic–acute, and acute cocaine, chronic desipramine, chronic fluvoxamine, chronic morphine, and chronic CNTF and BDNF infusions (see Materials and Methods). Data are expressed as mean ± SEM percent of control (*$p < 0.05$ vs control by χ^2 test). The numbers of animals used without and with cocaine, respectively, are chronic (12, 12), chronic–acute (8, 8), and acute (5, 5); without and with desipramine (5, 5); without and with fluvoxamine (5, 5); without and with morphine (4, 4); without and with CNTF (4, 5); and without and with BDNF (4, 4).

Effect of cocaine on functional responsiveness to CNTF in the VTA

Upon JAK2 phosphorylation and activation, STAT proteins associate with the CNTF receptor complex, undergo phosphorylation, and dimerize to form a functional transcription factor complex (see introductory remarks). STAT dimers then translocate to the nucleus where they regulate the expression of specific genes, one example of which is *c-fos* (Angel and Karin, 1991; Fu and Zhang, 1993; Coffer et al., 1995).

To assess the functional significance of the cocaine-induced increase in JAK2 immunoreactivity in the VTA, gel shift assays were used to measure the binding of STAT proteins to a mutant version of the sis-inducible element (mSIE) from the c-fos promoter. In this assay, mSIE binds STAT proteins with a higher affinity as compared with the wild-type SIE because of two point mutations (Wagner et al., 1990). Levels of STAT binding in the VTA were at barely detectable levels under control conditions, and previous chronic administration of cocaine had no detectable effect on this measure (Fig. 3*B*). This finding suggests that upregulation of JAK2 immunoreactivity by cocaine is not associated with an increase in basal levels of STAT binding.

We next assessed the influence of acute CNTF infusion into the VTA on STAT DNA binding activity. At doses between 0.08 and 0.5 μg, CNTF produced a strong, dose-dependent increase in STAT binding activity in the VTA (Fig. 3*A*). The identity of the induced bands as STAT dimers was confirmed by supershift assays (Fig. 3*D*; also see Materials and Methods). Increases in STAT binding in the substantia nigra were obtained only with the 0.5 μg dose of intra-VTA CNTF infusions (data not shown). This could reflect the spread of the higher dose of CNTF into neighboring regions, although such spread has not been investigated directly. Interestingly, previous chronic treatment of rats with cocaine dramatically increased the ability of CNTF to induce STAT binding in the VTA, an effect most apparent at lower CNTF doses (Fig. 3*C*).

To provide further evidence that cocaine-induced increases in JAK2 levels are associated with potentiated physiological responses to CNTF in the VTA, we studied c-Fos induction as an additional endpoint. We first established a dose of CNTF that when infused acutely into the VTA induces c-Fos expression. Infusion of 0.5 μg of CNTF produced a dramatic induction of c-Fos and several Fos-like proteins in control rats (Fig. 4*A*), whereas infusion of 0.1 μg produced a minimal effect (Fig. 4*B*). We next studied the effect of previous cocaine exposure on CNTF induction of c-Fos and related proteins using the lower dose of CNTF. As shown in Figure 4*B*, animals that had received chronic cocaine treatment (and were used 1 d after their last cocaine injection) exhibited a significant induction of c-Fos and related proteins after the CNTF infusion. This is in contrast to the lack of induction seen in control animals. This effect was not seen in animals that received a single injection of cocaine 1 d before the infusion of CNTF at a dose of 0.1 μg (data not shown). Note that chronic cocaine treatment, without CNTF infusions, did not alter basal levels of c-Fos or related proteins in the VTA as observed previously (Nye et al., 1995).

Immunohistochemical localization of JAK2 in the VTA

CNTF α receptors and the JAK–STAT pathway have been localized to both neurons and glia, although the cellular localization of these proteins within the VTA has not yet been reported. As a first step to determine whether the cocaine-induced upregulation of JAK2, and the subsequent hyper-responsiveness of the system

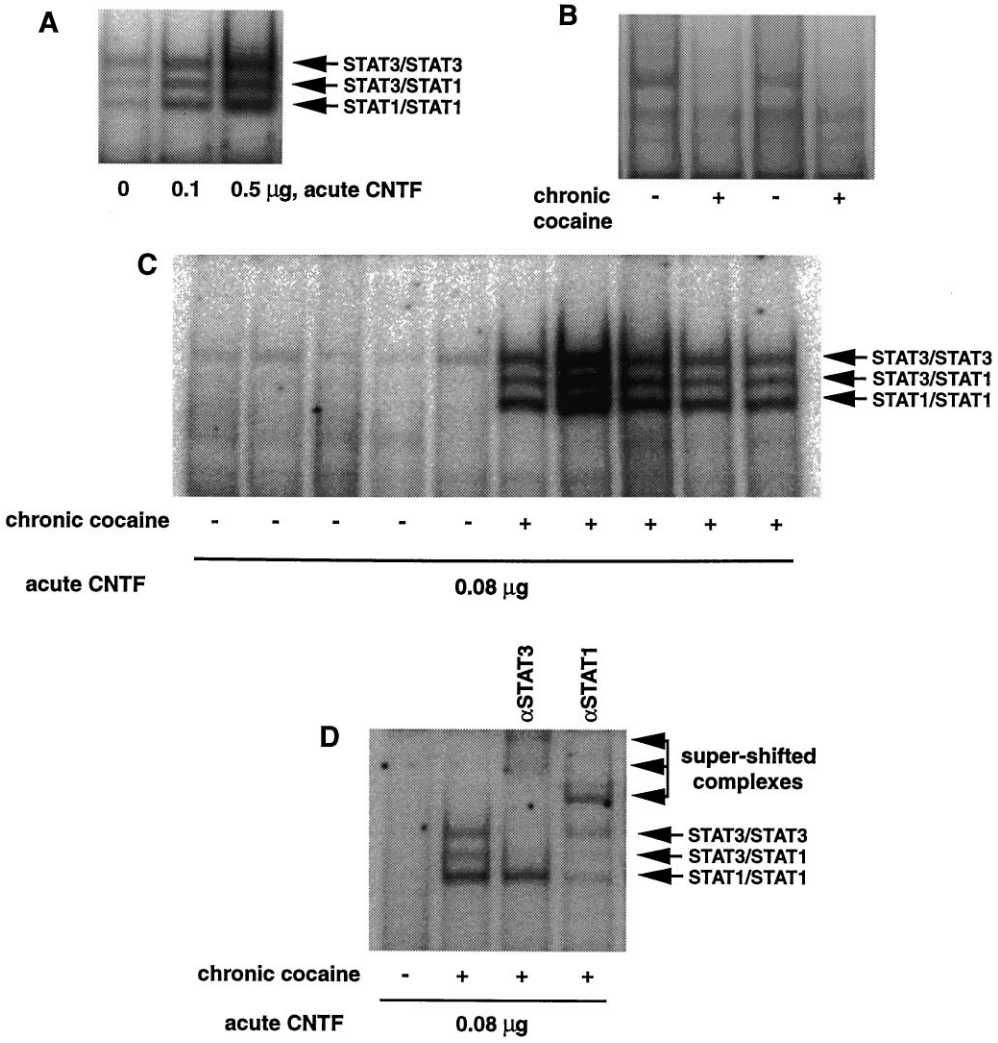

Figure 3. Regulation of STAT DNA binding activity in the VTA by cocaine and CNTF. *A*, Representative autoradiogram of STAT binding in the VTA 90 min after vehicle (0 μg) or CNTF (0.1 or 0.5 μg) was infused into this brain region of control rats. Four animals were used at each dose with equivalent results. *B*, Representative autoradiogram of STAT binding in the VTA under basal conditions (i.e., in the absence of intra-VTA infusions) in chronic saline (−)- or chronic cocaine (+)-treated rats. The figure shows an overexposed autoradiogram; there was no consistent effect of cocaine on the intensity of the very low levels of STAT binding apparent under basal conditions. *C*, Representative autoradiogram of STAT binding in the VTA 90 min after CNTF (0.08 μg) was infused into this brain region of chronic saline (−)- or chronic cocaine (+)-treated rats. Nine animals were used in each treatment group with equivalent results in two separate experiments. The figure illustrates the type of inter-animal variability seen in the magnitude of STAT binding induced by CNTF in chronic cocaine-treated rats. *D*, Representative autoradiogram of STAT binding analyzed by supershift assays. VTA extracts from a chronic saline (−)- and a chronic cocaine (+)-treated rat from *C* were used. The identification of the bands as STAT3 homodimers, STAT3:1 heterodimers, or STAT1 homodimers is based on the ability of anti-STAT3 (αSTAT3) and anti-STAT1 (αSTAT1) antibodies to supershift the various bands (see Materials and Methods).

to CNTF, is occurring in neurons or glia in the VTA, we analyzed the distribution of JAK2-like immunoreactivity in this brain region by double-labeling immunohistochemical techniques. The VTA has a relatively well characterized neuronal architecture. Dopaminergic neurons constitute ~60-80% of VTA neurons and GABAergic neurons constitute ~15-30% (Kalivas, 1994). We used TH immunoreactivity as a marker for VTA dopaminergic neurons and GFAP as a marker for astrocytes. The specificities of the anti-JAK2, anti-TH, and anti-GFAP antibodies were established on the basis of dilution ratios, competition experiments, deletion of the primary antibody from the experiment, and the expected regional distribution of the various proteins (see Materials and Methods).

As shown in Figure 5*A*, JAK2 immunofluorescence (red-yellow staining) was found to colocalize with TH-containing neurons (green staining) as well as non-TH-containing cells. Additionally, a small subset of TH-positive neurons did not colocalize with JAK2 immunoreactivity. To determine whether the non-TH-containing cells in the VTA that show prominent JAK2 immunolabeling are neurons or glia, colocalization with GFAP was studied. Figure 5*C* illustrates the colocalization of JAK2 within a subset of GFAP-positive cells; however, only a small fraction of the strongly labeled JAK2-containing cells (red-yellow staining) are positive for GFAP (green staining). Together, these findings suggest that JAK2-like immunoreactivity in the VTA also is likely to be present in nondopaminergic neurons.

Chronic administration of cocaine resulted in a detectable increase in overall JAK2-like immunoreactivity in the VTA, consistent with our immunoblotting data. This increase was most apparent in TH-containing neurons: as shown in Figure 5*B* (as compared with 5*A*), a larger number of TH-containing neurons co-labeled for JAK2 and with greater intensity (yellow vs red). There also seemed to be an increase in JAK2 immunoreactivity in GFAP-positive cells: labeling of JAK2 was more intense (yellow vs red) under cocaine-treated conditions (Fig. 5*D* as compared with 5*C*). The overall distribution pattern of JAK2 immunoreactivity, however, was not altered in brain sections from rats that had received chronic cocaine treatment, in that JAK2 immunoreactivity was distributed among TH-containing neurons, GFAP-positive cells, and other cell types as seen under control conditions. Levels of TH immunoreactivity also seemed to be increased by chronic cocaine treatment in the VTA, consistent with increases observed previously by immunoblotting (see introductory remarks).

DISCUSSION

In a previous study, we demonstrated that chronic intra-VTA infusions of CNTF, but not of other neurotrophic factors, mim-

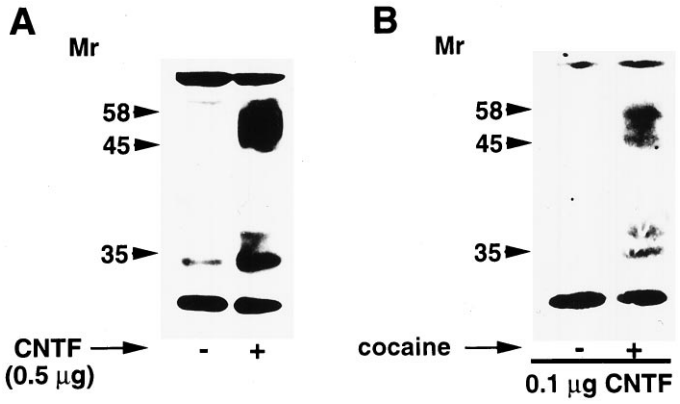

Figure 4. Regulation of c-Fos and related proteins in the VTA by cocaine and CNTF treatments. *A*, Representative autoradiograms of c-Fos (58 kDa) and related proteins in the VTA 3 hr after vehicle or CNTF (0.5 μg) was infused into this brain region of control rats. Four animals were used in each treatment group with equivalent results. *B*, Representative autoradiograms of c-Fos and related proteins in the VTA 3 hr after CNTF (0.1 μg) was infused into this brain region of chronic saline-treated and chronic cocaine-treated rats. Four animals were used in each treatment group with equivalent results. The specificity of the resulting immunoreactivity was demonstrated by its blockade by preabsorption of the antibody with purified M-peptide antigen (Hope et al., 1994).

icked the effects of chronic cocaine and chronic morphine treatments on specific biochemical endpoints (e.g., regulation of TH and GFAP) in the VTA (Berhow et al., 1995). These findings raised the possibility that long-term exposure to these drugs of abuse could conceivably produce these adaptations, at least in part, via regulation of the signal transduction pathway used by CNTF. In the present study, we provide direct evidence for such regulation, namely, induction of JAK, the major effector for CNTF. We show that chronic administration of cocaine increases levels of JAK2, but not JAK1, immunoreactivity in the VTA. In contrast, acute administration of cocaine failed to produce this effect. Cocaine regulation of JAK2 immunoreactivity in the VTA also showed regional specificity, in that it was not observed in the other brain regions that were examined.

In addition, cocaine regulation of JAK2 showed pharmacological specificity. Upregulation of JAK2 was not seen in response to chronic administration of desipramine (a norepinephrine reuptake inhibitor) or fluvoxamine (a serotonin reuptake inhibitor). Although these data support an important role for dopamine reuptake blockade in cocaine regulation of JAK2, further experiments are required to establish this with certainty. Chronic administration of morphine also failed to regulate JAK2 levels in the VTA. This is somewhat surprising given the many common, chronic actions of cocaine and morphine in this brain region (Nestler, 1992; Nestler et al., 1993). On the other hand, cocaine and morphine are known to differentially affect both dopaminergic and nondopaminergic neurons in this brain region in many ways (Henry et al., 1989; Johnson and North, 1992; Nestler et al., 1993), which could be related to their differential effects on JAK2.

The cocaine-induced increase in JAK2 levels in the VTA was shown to be associated with enhanced physiological responsiveness to CNTF as measured by STAT DNA binding activity and c-Fos induction, two known functional endpoints of JAK activation (see introductory remarks). Acute infusion of CNTF into the VTA, at doses that had no detectable effect in saline-treated animals, was found to produce a dramatic increase in STAT binding activity and in c-Fos induction in rats that had been treated chronically with cocaine. In contrast, there was no detectable increase in STAT binding activity or c-Fos induction under basal conditions in chronic cocaine-treated rats. These findings suggest that the elevated levels of JAK2 seen under cocaine-treated conditions are not tonically activated but rather provide for an increased responsiveness of the system to CNTF and perhaps related cytokines.

Immunohistochemical techniques were used to study the cellular localization of JAK2 within the VTA. Consistent with previous studies that focused on other brain regions (Ip and Yancopolous, 1996), we found that JAK2-like immunoreactivity is localized to both neurons and glia in the VTA; specifically, JAK2-like immu-

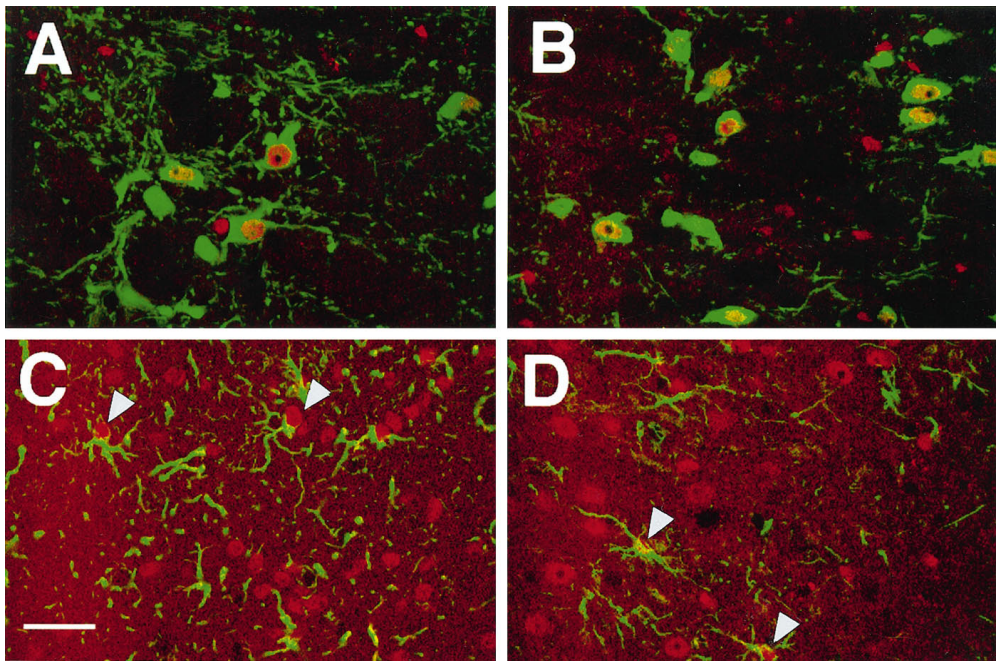

Figure 5. Localization of JAK2 within TH- and GFAP-containing cells in the VTA using double-labeling immunohistochemical techniques. *A* and *B* show representative pictures of JAK2 (red-yellow) colocalized within TH-containing neurons (green). *C* and *D* show representative pictures of JAK2 (red-yellow) colocalized within GFAP-containing astrocytes (green). Sections were obtained from chronic cocaine-treated (*B, D*) and chronic saline-treated (*A, C*) animals. In *C* and *D*, double-labeled cells are indicated by *white arrowheads*. The results are representative of the analysis of multiple sections of five rats in each treatment group. Note that although the size of JAK2 immunolabeled cells in general appears larger under cocaine-treated conditions, no conclusion about cell size is possible with the methodologies that were used. Scale bar, 25 μm.

noreactivity was found to colocalize within TH-positive neurons, GFAP-positive astrocytes, and other cells types, presumably certain nondopaminergic neurons. A cocaine-induced increase in JAK2 levels was clearly apparent in TH-positive neurons as well as in non-TH-containing cells, including glia. The mechanism by which chronic cocaine exposure regulates JAK2 levels in glial cells and nondopaminergic neurons remains unknown, but conceivably could involve effects of the VTA dopamine neurons (widely believed to be the initial target of cocaine) on the other cell types in this region achieved either directly (via some cell–cell interaction) or indirectly (e.g., via regulation of the hypothalamic–pituitary–adrenal axis known to be activated by cocaine).

Additional work is now needed to identify the adaptations induced in the VTA by cocaine that are mediated via induction of JAK2 in these various cell types. For example, CNTF increases TH expression in dopaminergic neurons *in vivo* and *in vitro* (Hagg and Varon, 1993; Louis et al., 1993a; Magal et al., 1993; Berhow et al., 1995; Rabinovsky et al., 1995), raising the question of whether the cocaine-induced increase in TH levels in the VTA is mediated via upregulation of the JAK–STAT pathway. A related question raised by the present findings is whether chronic cocaine exposure increases levels of CNTF or a related cytokine in this brain region. Increased levels of such a cytokine could underlie the upregulation of JAK2. We observed that acute exposure to CNTF activates the JAK–STAT pathway, whereas repeated exposure to CNTF upregulates JAK2 and thereby further increases the functional responsiveness of the system to CNTF or a related cytokine. In this way, upregulation of JAK2 and the JAK–STAT pathway could represent one potential mechanism of the sensitizing actions of repeated cocaine exposures. Of course, cocaine regulation of the JAK–STAT pathway is likely just one of several mechanisms by which chronic cocaine exposure produces long-term changes in the VTA. As just one example, chronic cocaine (and also chronic morphine) treatment has been shown recently to activate the extracellular signal-regulated kinase signaling cascade, another neurotrophic factor-regulated pathway, in this brain region (Berhow et al., 1996).

The studies reported here provide the first evidence for pharmacological regulation of the JAK–STAT pathway, and its regulation by CNTF, in the brain. The observed regulation occurs within a discrete brain region whose role in the regulation of motivational behavior has been well characterized. In this way, cocaine regulation of JAK2 levels and the functional activity of the JAK–STAT pathway in the VTA provide a model system in which cytokine regulation of neuronal functioning can be delineated at a molecular level in a physiologically relevant, *in vivo* setting. The studies reported here also highlight the novel types of mechanisms that must be considered in explaining the long-term adaptations of the brain to cocaine and perhaps other chronic perturbations.

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