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Dopaminergic and Glutamatergic Mechanisms Mediate the Induction of FOS-Like Protein by Cocaethylene

JUDITH M. HOROWITZ, JEAN M. DiPIRRO, MARK B. KRISTAL AND GERMAN TORRES[1]

Behavioral Neuroscience Program, Department of Psychology, State University of New York at Buffalo, Buffalo, NY 14260-4110

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ABSTRACT: Cocaethylene is a psychoactive metabolite formed during the combined consumption of cocaine and ethanol. As this metabolite has many properties in common with cocaine, it is conceivable that cocaethylene administration may induce the activity of nuclear transcription factors that regulate the expression of late-response genes. Therefore, the temporal induction of FOS-like protein in rat brain was examined following IP administration of 60 μmol/kg cocaethylene. Immunoreactivity for the protein was detectable at 1 h in striatal neurons and had virtually disappeared 6 h after drug treatment. Administration of specific dopaminergic (SCH-23390; 0.5 mg/kg) and glutamatergic (MK-801; 1 mg/kg) receptor antagonists prior to cocaethylene indicated a significant role for dopamine (D_1) and *N*-methyl-D-aspartate receptor subtypes in mediating the nuclear induction of the aforementioned transcription factor protein. In contrast, no significant effects on FOS-like protein in discrete neurons of the caudate putamen were found when spiradoline (U-62066), a kappa opioid-receptor agonist, was administered either IP (10 mg/kg) or directly (50 nmol) into the brain parenchyma. In addition, we uncovered a differential sensitivity of Long–Evans rats to the behavioral effects of cocaethylene, with the psychoactive metabolite producing significantly less behavioral activity (e.g., locomotion, rearing, and continuous sniffing) than that produced by cocaine (molar equivalent of 60 μmol/kg cocaethylene). These findings indicate both common and disparate effects of cocaethylene and its parent compound, cocaine, on receptor pathways that regulate target alterations in gene expression and drug-induced motor behavior. © 1997 Elsevier Science Inc.

KEY WORDS: Cocaine, Dopamine, D_1 receptor, Dynorphin, Ethanol, Locomotor activity, NMDA receptor, Rat, Serotonin, Spiradoline.

INTRODUCTION

Cocaethylene is an ethyl metabolite of cocaine formed by a liver carboxylesterase in the presence of ethanol. It is detected in urine and plasma of humans tested positive for both drugs [7] and in liver and brain from decedents of cocaine overdose [7,22,28]. The simultaneous use of cocaine and ethanol is of particular concern as this pattern of drug use may exacerbate neurochemical and behavioral disturbances associated with compulsive drug craving [21]. Cocaethylene may potentiate such disturbances because it possesses neurochemical and psychotropic effects comparable to those of cocaine. For example, cocaethylene has a high affinity for the dopamine (DA) transporter site [7], thereby increasing extracellular DA concentrations in the nucleus accumbens of the mammalian brain [11]. Furthermore, cocaethylene exposure elicits locomotor activity and acts as a reinforcer in self-administration studies in nonhuman primates and rats [12,14,29]. The apparent similarity of action by cocaine and cocaethylene on dopaminergic neurons may indicate why combined use of cocaine and ethanol is a widespread pattern of drug abuse. The in vivo formation of cocaethylene may contribute significantly to the effects of polydrug abuse by prolonging cocaine-mediated euphoria.

Recently, we reported [35] that cocaethylene also exerted qualitatively similar effects on gene expression as those ascribed to cocaine and other psychostimulant drugs [3,39,40]. Systemic administration of cocaethylene to rats evoked a marked induction of FOS, a transcription factor protein, in numerous nerve cells of the caudate putamen and nucleus accumbens (collectively referred to as the striatum). FOS and other related proteins are known to form a variety of homo- and heterodimers that bind with high affinity and specificity to AP-1 DNA sequences (TGACTCA), thereby stimulating the transcription of genes postsynaptic to dopaminergic input such as prodynorphin and proenkephalin [20,27,30,32]. Such alterations in gene expression may form the underlying intrinsic mechanism(s) for drug addiction. Several studies have demonstrated that induction of FOS and other gene products by cocaine and related psychostimulants is mediated by D_1 and *N*-methyl-D-aspartate (NMDA) receptors [3,19,31,33,39,40], as blockade of these membrane-bound elements prevents gene expression in drug-sensitive neural circuits. The present set of studies was therefore undertaken to characterize (a) the temporal patterns of FOS induction following acute treatment of cocaethylene, and (b) the postsynaptic receptors mediating such nuclear induction. To accomplish these goals, we used well-known receptor antagonists such as SCH-23390 and MK-801 to identify basic intracellular mechanisms that might promote mRNA synthesis of target genes in rat striatum.

METHODS

Animals

Female Long–Evans rats (220–300 g) were used in these studies. The reason for using females is that we have failed to

[1] To whom requests for reprints should be addressed.

detect sex differences in immediate-early gene induction in discrete neurons of the caudate putamen after cocaine administration [34]. In addition, no attempt was made to establish the state of the estrous cycle in the rats. The animals were housed three per cage and maintained on a 12 h light:dark cycle with free access to food and water. Cocaethylene hydrochloride (Research Biomedicals Inc., Natick, MA) was dissolved in sterile saline and injected IP at a dose of 60 μmol/kg. This concentration corresponds to an approximate dose of 20 mg/kg cocaine; a moderate dose known to result in a significant induction of FOS-like protein in striatal perikarya, and increased locomotor activity in rodents [36,38]. Behavioral activation of rats exposed to cocaethylene was assessed according to the behavioral rating scale of Kalivas et al. [13]. This scale provides an excellent estimate of behavioral activity ranging from exploratory behavior to stereotypy [1]. The behaviors were rated 5 min before the cocaethylene injection and every 5 min thereafter for 30 min by an observer ''blind'' to the experimental conditions. At every observation time, a single score was given on a scale from 1–10, where 1 = asleep or still; 2 = inactive or in-place activity; 3 = locomotion (all four feet moving within a 10 s period), rearing or sniffing ($>$ 3 s duration); 4 = any combination of two of locomotion, rearing or sniffing; 5 = continuous sniffing for 10 s without locomotion or rearing; 6 = continuous sniffing for 10 s with locomotion or rearing; 7 = patterned sniffing for 5 s; 8 = patterned sniffing for 10 s; 9 = continuous gnawing; 10 = bizarre diskinetic movements or seizures. It should be noted that we never observed scores greater than 6 in the present study. To compare the behavioral effects of cocaethylene to those of cocaine, a subset of female Long–Evans rats were injected IP with cocaine HCl (Sigma; molar equivalent of 60 μmol/kg cocaethylene) and their behaviors rated as previously described. Behavioral data were evaluated by a two-way ANOVA with repeated measures on one factor followed by Newman–Keuls post hoc test comparisons. All testing and surgical procedures were carried out in accordance with the *NIH* Guide for the Care and Use of Laboratory Animals, and with approval from the University at Buffalo IACUC.

Experimental Procedures

In the first set of experiments, we assessed the temporal induction of FOS-like protein following IP administration of cocaethylene. One, 6 or 12 h after the injection, the rats (n = 3/time period) were deeply anesthetized with sodium pentobarbital and then perfused via the ascending aorta with heparinized saline followed by 4% paraformaldehyde in Na-phosphate buffer for 15 min. In the second set of experiments, rats (n = 3/group) were injected IP with either saline (1 ml/kg), SCH-23390 (0.5 mg/kg; a selective D_1 receptor antagonist) or MK-801 (1 mg/kg; a highly potent and selective noncompetitive NMDA receptor antagonist). All antagonist drugs (Research Biomedicals Inc., Natick, MA) were dissolved in sterile saline and prepared on the day of administration. Thirty minutes after each appropriate injection the rats received a single cocaethylene injection and were perfused 1 h later. The doses for SCH-23390 and MK-801 were selected on the basis that they block the induction of *c-fos* mRNA and its encoded protein in rat brain after the administration of cocaine or amphetamine [3,38].

It has been reported that the striatal dynorphin opioid system may play a significant role in the induction of *c-fos* by cocaine because kappa opioid-receptor agonists block such induction [33]. To investigate this phenomenon further and to characterize additional receptor systems mediating the induction of FOS-like protein by cocaethylene, we administered spiradoline mesylate (U-62066; a highly selective kappa opioid-receptor agonist) to rats 15 min prior the IP injection of cocaethylene. Spiradoline (Research Biomedicals Inc.) was dissolved in 0.02% ascorbic acid and injected IP or directly into the brain parenchyma at a dose of 10 mg/kg or 50 nmol, respectively. Control rats (n = 3/group) received ascorbic acid with no spiradoline added. The time course and drug doses were those described by Steiner and Gerfen [33]. One hour after cocaethylene exposure, the rats were deeply anesthetized and perfused as previously described.

Surgical Procedures

Microinfusions of spiradoline or ascorbic acid were placed unilaterally into the right caudate putamen through burr holes placed in the rat skull (n = 4). All stereotaxic coordinates (relative to bregma) were based on the rat brain atlas of Paxinos and Watson [26]. Briefly, a guide cannula was implanted into the caudate putamen (AP = +0.40 mm; ML = 3.0 mm; DV = −4.0 mm) and anchored to screws in the skull with acrylic cement. To prevent clogging of the cannula, a dummy cannula of the same length was inserted for 14 days as the animals recovered from the surgery. To minimize the risk of unrelated stress effects, the rats were handled and accustomed to the microinfusion procedure for 5 days prior to the study. On the day of testing, the dummy cannula was replaced with another that extended 1.0 mm beyond the tip of the guide cannula and spiradoline microinfused with a pump at a volume of 1 μl (0.2 μl/min for a total time of 5 min).

Immunocytochemistry

The immunocytochemical methods for the detection of FOS-like protein have been described in detail previously [38] and are only briefly summarized below. Free-floating brain sections (40 μm) ranging from plates 15 (bregma 0.70 mm; including rostral components of the caudate putamen) to 26 (bregma −2.12 mm; including the paraventricular nucleus of the hypothalamus) were incubated for 48 h with a rabbit polyclonal IgG raised against the *N*-terminal (residues 4–17) of the rodent $P62_{c\text{-}fos}$ (Oncogene Science). The antibody was diluted 1:1000 in KPBS with goat serum and Triton X-100. The specificity of the antiserum for FOS has been demonstrated by the fact that FOS detection in brain is abolished by preabsorption with synthetic peptides [9]. However, crossreactivity of FOS-like protein with related proteins cannot be ruled out. To quantify the results, brain sections positive for FOS-like protein were viewed through an Olympus microscope equipped with a (10 mm^2) grid reticule at a magnification of 20×. We selected a caudal component of the caudate putamen (bregma −0.26 mm) as the area for microscopic analysis because the number of immunoreactive perikarya after cocaine exposure is higher in caudal than in rostral components of the caudate putamen. No correction factor was employed in these studies, and, therefore, there is the possibility that positive perikarya may have been counted twice. The data were analyzed first by ANOVA, and then by Student–Newman–Keuls tests for between-group means, with statistically significant differences defined as $p < 0.05$.

RESULTS

Cocaethylene exposure resulted in numerous clusters of perikarya positive for FOS-like protein across the dorsal-central quadrant of the caudate putamen. Localization of the transcription factor protein in this telencephalic region was specifically neuronal and nuclear. Based upon our time-course studies, induction of FOS-like protein by cocaethylene was rapid and transient, peaking between 1 and 2 h and returning to basal levels

within 6 h (Fig. 1). Indeed, statistical analysis showed a significant difference across time when all three groups were compared, $F(2, 8) = 82.3$, $p < 0.01$. In addition, pairwise multiple comparison analysis revealed no marked differences between rats sacrificed at 6 and 12 h, $t(4) = 1.67$, $p > 0.28$. Although cocaethylene also induced FOS-like protein in the nucleus accumbens, such induction was low and highly variable, thereby preventing reliable comparisons of the number of nerve cells bearing the protein signal among striata from different rats. As predicted from previous studies [3,38], rats treated with saline-vehicle injections showed no immunoreactivity of FOS in caudate putamen or nucleus accumbens perikarya (Fig. 2).

The involvement of D_1 and NMDA receptor subtypes in the mediation of FOS-like protein by cocaethylene was confirmed: induction of the nuclear protein was markedly blunted by SCH-23390, $t(4) = 9.4$, $p < 0.0008$, and MK-801, $t(4) = 9.1$, $p < 0.0007$, relative to rats injected with the combined saline and cocaethylene treatment. As seen in Fig. 2, both receptor antagonists exerted almost complete suppression of FOS-like protein in striata derived from cocaethylene-treated rats. According to post hoc analysis, this suppression was of equal magnitude and confined to similar anatomical sites as no statistically significant differences ($p > 0.05$) were noted in these parameters between the two drugs. Induction of FOS-like protein by cocaethylene, however, was not significantly affected ($p > 0.8$) by spiradoline pretreatment (Fig. 3). This finding diverges from that of an earlier report emphasizing the involvement of striatal dynorphin opioid systems in the induction of *c-fos* by cocaine [33]. This phenomenon, moreover, was observed in both IP and intraparenchymal preparations, thereby ruling out the possibility of indirect kappa opioid-receptor mediated effects on striatal FOS. It is noteworthy that systemic administration of spiradoline resulted in the induction of FOS-like immunoreactivity, particularly along the lateral ventricles and dorsal-lateral aspects of the caudate

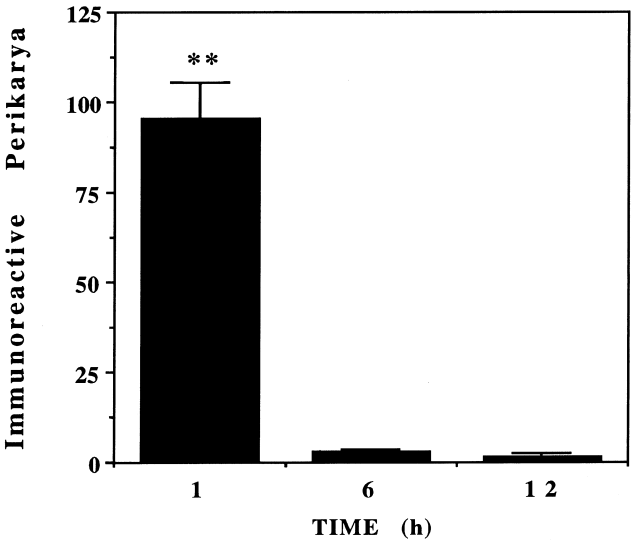

FIG. 1. Temporal effects of cocaethylene (60 μmol/kg) on striatal FOS-like protein. Each bar represents the mean ± SEM of positive immunoreactive neurons obtained from brains of rats sacrificed at depicted time intervals. $^{**}p < 0.01$. It should be noted that ethanol blocks the induction of the transcription factor protein by cocaine [34,35] and, therefore, cocaethylene may have an impact on striatal immediate-early gene expression only after ethanol has been absorbed by peripheral surfaces or undergone oxidative metabolism into acetaldehyde and hydrogen ions.

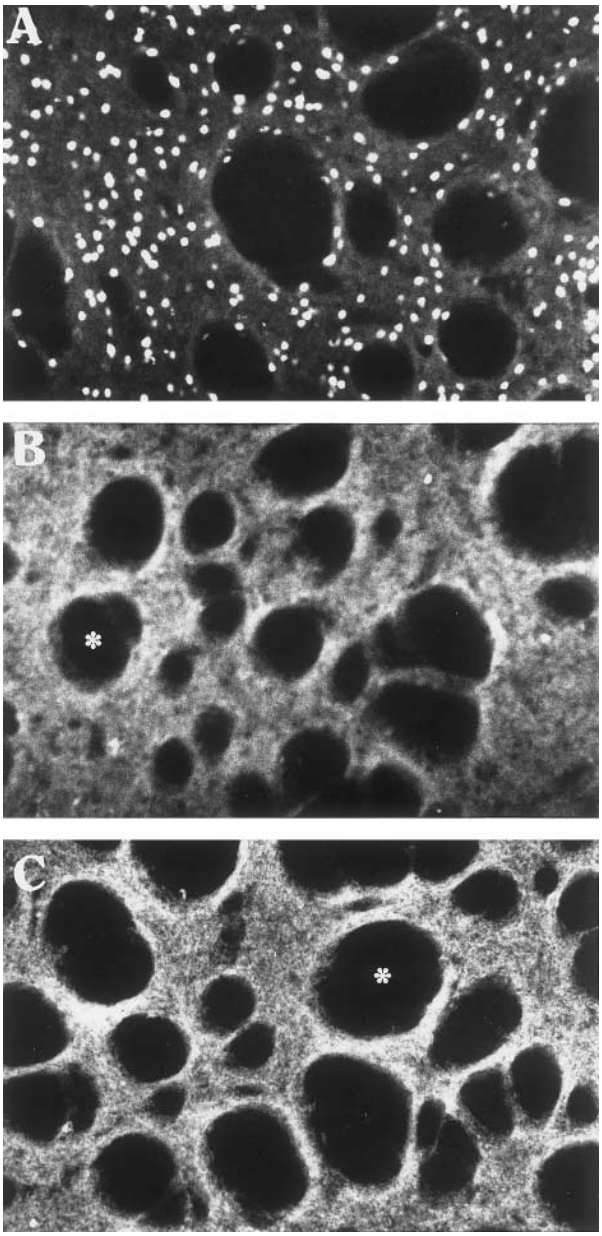

FIG. 2. Reverse-image photomicrographs depicting the pretreatment effects of (A) saline, (B) SCH23390, or (C) MK-801 on cocaethylene-induced FOS-like protein in caudate putamen perikarya. Blockade of DA and glutamate receptors by the aforementioned drugs reduced further the incidence of behavioral activity in Long–Evans rats (i.e., a rating score of 1 = still or 2 = inactive or in-place activity). *Unreactive fascicles of myelinated fibers coursing through the caudate. Original magnification, ×20.

(Fig. 4). In addition, spiradoline produced a noticeable reduction of locomotor activity along with body flattening in rats: prototypical behavioral indices ascribed to spiradoline [18,33]. Therefore, spiradoline exerted its predicted behavioral effects on Long–Evans rats but failed to prevent the induction of FOS-like protein by cocaethylene.

Interestingly, cocaethylene treatment elicited only modest locomotor activity in Long–Evans rats, but stereotypic motor behaviors (e.g., rearing, sniffing, and head bobbing) were not par-

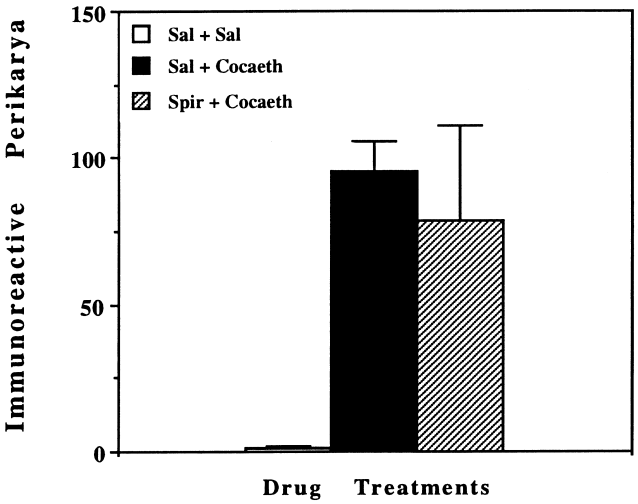

FIG. 3. Effects of spiradoline (Spir) on cocaethylene (Cocaeth)-induced FOS-like protein in medium-sized spiny neurons. Each bar represents the mean ± SEM of positive immunoreactive perikarya from brains of rats pretreated IP with saline (Sal) or the aforementioned kappa opioid-receptor agonist. Note that although there was a trend toward a decrease in the number of immunopositive neurons, the overall blockade of the protein did not approach that observed for SCH23390 or MK-801. If there are subtle, discrete changes in gene expression as a result of spiradoline pretreatment, the immunocytochemical methods used in the present studies failed to detect them.

ticularly noticeable at the 60 μmol/kg dose (Fig. 5). In contrast, female rats injected (IP) with cocaine exhibited a robust sequeale of behaviors that was characterized by locomotion, exploration, or rearing in one place and continuous sniffing (i.e., a rating score of 6). Indeed, a two-way repeated ANOVA on one factor demonstrated a drug, $F(1, 42) = 215.5$, $p < 0.01$, time, $F(6, 42) = 45.1$, $p < 0.01$, and drug × time interaction, $F(6, 42) = 33.8$, $p < 0.01$). Further, analysis of the time course showed that cocaine produced a more sustained increase in locomotor activity than cocaethylene, as indicated by the presence of strong motor behavior by the end of the test session. This is of interest because (a) it suggests that Long–Evans rats show a differential sensitivity to the behavioral effects of cocaethylene and cocaine; and (b) it points to the possibility that Long–Evans rats may differ in their motoric output to other strains as we recently reported that the same cocaethylene dose elicited significant locomotor activity in Sprague–Dawley rats [35]. These data indicate that rat phenotypes may differ in their behavioral responses to cocaethylene.

DISCUSSION

The present results show that cocaethylene, an active byproduct of the interaction between ethanol and cocaine, induced the neuronal expression of FOS-like protein within drug-sensitive brain structures. This transcription protein may regulate subsequent wave patterns of gene expression, thereby mediating long-term consequences of transsynaptic stimulation. Induction of FOS-like protein by cocaethylene was apparent within 1 h after treatment and had an extended time course of about 6 h. Therefore, the rise in protein levels is rapid and relatively transient in duration. In comparison, cocaine effects on transcriptional levels of FOS (and its mRNA) are of longer duration, as they remain elevated 6–24 h after drug exposure [24,40]. This may indicate that although cocaethylene and cocaine are equipotent at initiating cellular transcription, cocaine may be more potent than cocaethylene in prolonging the responses of the target neuron to transsynaptic signals. In this context, differences between cocaethylene and cocaine are emerging that could explain, in part, our present results. For example, cocaethylene is less potent than cocaine at blocking striatal serotonin (5-HT) reuptake in vivo [5,6]. This may be of importance because there is evidence that, in addition to the dopaminergic system, the serotonergic system also plays a prominent role in the induction of *c-fos* and *zif/268* mRNA by cocaine [4]. Perhaps the rapid shutoff of FOS-like protein after cocaethylene administration may reflect 5-HT input that is too low to sustain bursts of cellular activity from DA. Moreover, route-dependent differences in brain bioavailability between cocaethylene and cocaine have been observed in microdialysis studies [5,6], which may impact the relative potency of each drug with respect to the time and magnitude of their effects on transcription factor proteins.

The effects of the receptor-selective DA antagonist SCH-23390 indicate that D_1 receptor subtypes in the striatum mediate

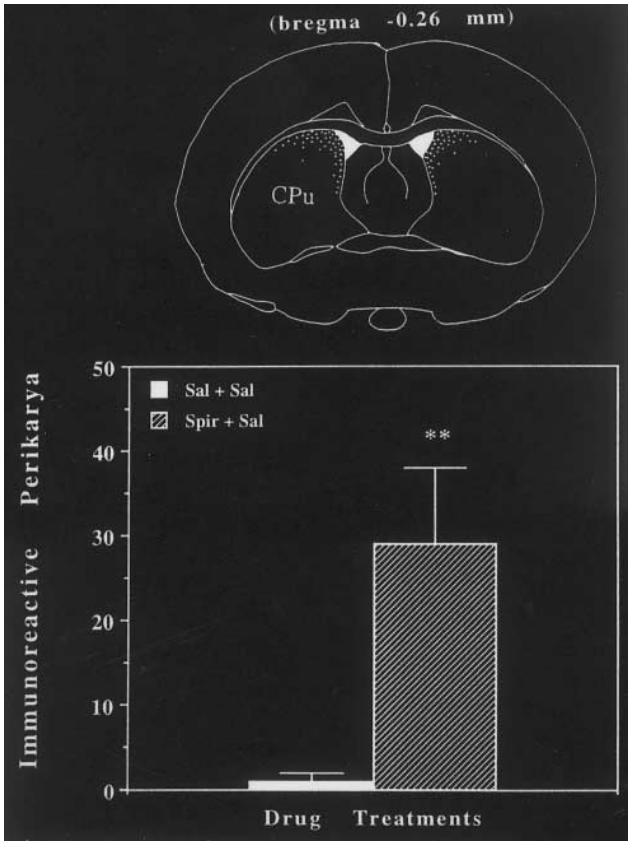

FIG. 4. Effects of spiradoline (Spir) or saline (Sal) on FOS-like protein in nerve cells of the caudate putamen (CPu). Diagrammatic figure (top) and mean ± SEM of perikarya immunoreactive positive for the protein (bottom). Spiradoline injected IP (10 mg/kg) evoked a specific regional pattern of FOS-like protein that was confined along the ependymal wall that lines the lateral ventricles and in dorsal-lateral aspects of the CPu. Intrastriatal infusion (50 nmol) of spiradoline also resulted in a modest induction of the transcription factor protein, without causing behavioral effects by itself. In addition, there was indication of scarring and gliosis in the vicinity of the cannula placement that coincided with the regional pattern of FOS-like immunoreactivity. $^{**}p < 0.05$ when compared to saline-treated rats. $n = 3–4$ rats per group.

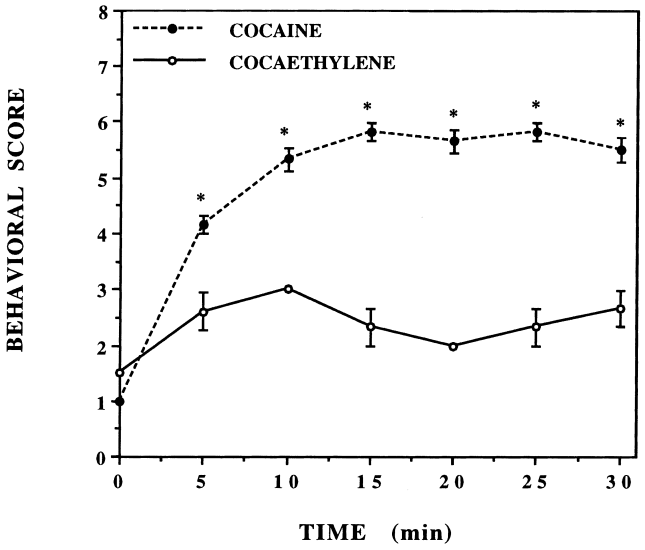

FIG. 5. Differential effects of cocaethylene and cocaine on behavioral activation in female Long–Evans rats (n = 4–6 per group). Values represent mean ± SEM. Behaviors were rated 5 min before the IP injection and every 5 min for 30 min after each drug treatment. See the Methods section for details on the behavioral rating scale. Although cocaethylene stimulated the expression of FOS in the striatum of intact rats, the incidence of behavioral activity (e.g., locomotion, rearing in one place, and continuous sniffing) was considerably less with this drug than in rats injected with the parent compound, cocaine. $*p < 0.05$ relative to cocaethylene-treated rats.

the effects of cocaethylene on FOS-like protein. Therefore, DA receptors coupled to adenylyl cyclase and cAMP-dependent protein kinase systems are likely to coordinate the activity of nuclear transcription factors [8]. For instance, this signaling amplification system phosphorylates cAMP-responsive element binding proteins (CREB) that, in turn, stimulate transcriptional initiation [23]. Indeed, D_1 receptor-mediated CREB phosphorylation appears to be an obligatory cellular event for the induction of *c-fos* by amphetamine [10,15] and perhaps by cocaethylene and cocaine as well. Our results also show that NMDA receptor activation is crucial for the induction of FOS-like protein by cocaethylene, as very little nuclear protein was detected in striata from rats pretreated with MK-801. These data suggest that DA and glutamate may act cooperatively in the striatum to exert common, long-term effects on cellular transcription, and further support the contention that NMDA receptor activation is necessary to trigger induction of *c-fos* via a calcium-requiring mechanism [17]. Taken together, these observations clearly indicate that cocaethylene and cocaine employ common postsynaptic receptor systems to impart a signal that regulates gene expression by modulating the activity of nuclear transcription factors.

With the recognition that DA and glutamate systems regulate the induction of transcriptional proteins and their target genes, the participation of additional receptor subtypes that could also account for the long-term effects of psychotropic drugs on striatal neurons has generated considerable interest. In this context, Steiner and Gerfen [33] reported that spiradoline suppressed cocaine-induced *c-fos* in the rodent striatum. This telencephalic region is densely populated with opioid receptor subtypes, with kappa opioid-receptor mRNA localized preferentially in medial and ventral components of the caudate putamen [20]. Spiradoline, however, failed to suppress the induction of FOS-like protein by cocaethylene despite the fact that both systemic and intrastriatal administrations of this drug were used to characterize the predominant receptor pathways that modulate translation of intercellular signaling events. Two separate (but not mutually exclusive) explanations can be offered to account for this discrepancy. First, cocaethylene may have a different pharmacological profile than that of cocaine with respect to its effects on brain kappa opioid-receptor systems. It could be argued that since cocaethylene is less effective than cocaine with respect to 5-HT reuptake, perhaps interactions of prodynorphin-derived opioid peptides with serotonergic as well as glutamatergic nerve cells are obligatory events underlying the effects of spiradoline on FOS-like protein. While alternative mechanisms cannot be ruled out, this hypothesis is testable and warrants particular attention. Second, the possibility exists of genotypic-dependent differences in sensitivity to cocaethylene. For instance, Long–Evans rats may differ from Sprague–Dawley rats (strain used by Steiner and Gerfen) in specific signal transduction pathways that regulate gene expression. In this respect, prominent differences have been found in levels of tyrosine hydroxylase (the rate-limiting enzyme in the biosynthesis of DA) and other phosphoproteins in brains of Lewis and Fischer strains that may underlie their differences in response to opiates, ethanol or cocaine [2,16,25]. It is noteworthy that we, too, noticed apparent genetic contributions to behavior associated with cocaethylene exposure. For example, Long–Evans rats showed relatively modest motoric effects to cocaethylene as compared to those of Sprague–Dawley rats [35,37]; this may indicate another inherent difference between these two genetically divergent populations of rats. In this context, it should be noted that Long–Evans rats exhibited a differential sensitivity to the behavioral effects of cocaethylene and cocaine. While cocaine produced locomotor activity followed by stereotypical behaviors (rearing in one place and continuous sniffing), cocaethylene failed to produce a similar pattern of behavioral responses. It is possible that the differences in the behavioral effects produced by cocaethylene and cocaine could be the result of strain differences (see above) or pharmacological differences between the two drug congeners. For instance, it is conceivable that changes in motor behavior depend on equivalent forebrain DA and 5-HT concentrations to produce a hyperactive state. Because cocaine, but not cocaethylene, significantly increases 5-HT levels in the striatum [5,6], this difference may in part be responsible for differences in behavioral activation. Studies addressing these issues are presently in progress.

Drug addiction is characterized by neural adaptations that may be related to changes in the activity of transcription factors. Proteins, like FOS, serve as messengers relaying information from the nerve cell membrane to the nucleus, thereby modulating subsequent patterns of gene expression. Cocaethylene, an active byproduct of the interaction between ethanol and cocaine, has been found to induce the transcription of *c-fos* in neural substrates linked to drug use and drug craving. The initial intracellular mechanism(s) mediating this nuclear induction was determined to be regulated, at least in part, by D_1 and NMDA receptor subtypes. Although opioid ligands such as dynorphin are capable of influencing the activity of dopaminergic neurons in rat striatum, a selective kappa opioid-receptor agonist failed to suppress the induction of FOS seen after cocaethylene administration. The mechanism(s) for this effect is not known, but may be due to differences in the pharmacological profile of cocaethylene and cocaine.

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