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Evidence for alterations in presynaptic serotonergic function during withdrawal from chronic cocaine in rats

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Abstract

The effects of repeated cocaine administration on serotonin (5-hydroxytryptamine, 5-HT) function were investigated by comparing the corticosterone response to 5-HT receptor agonists in cocaine-treated and vehicle-treated rats. Male rats were fitted with indwelling jugular catheters and received cocaine (15 mg/kg i.p., b.i.d.) or saline for 7 days. Rats were challenged with either saline, the 5-HT releaser fenfluramine (1.2 mg/kg i.v.), the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT; 50 μg/kg i.v.), or the 5-$HT_{2A/2C}$ receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; 100 μg/kg i.v.) 42 h and 8 days after the final chronic treatment. Repeated blood samples were withdrawn immediately before and at 15, 30 and 60 min after acute challenge injections. All 5-HT receptor agonists increased plasma corticosterone, but the fenfluramine-induced rise in corticosterone was significantly attenuated in cocaine-treated rats withdrawn for 42 h. This blunted response to fenfluramine exhibited only partial recovery when examined at 8 days postchronic treatment. Corticosterone responses to 8-OH-DPAT and DOI were not affected by cocaine exposure. Our data suggest that chronic cocaine produces deficits in presynaptic 5-HT function, and alterations in 5-HT neurotransmission may underlie the dysphoria experienced by abstinent cocaine users. Neuroendocrine challenge tests should be performed in human addicts to evaluate potential 5-HT dysfunction associated with cocaine abuse.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); Cocaine; Fenfluramine; Corticosterone, presynaptic

1. Introduction

It is generally accepted that mesolimbic dopamine neurons play a major role in cocaine addiction (Kuhar et al., 1991). However, an increasing number of studies indicate that serotonergic (5-hydroxytryptamine, 5-HT) mechanisms may also be involved in the reinforcing properties of cocaine (Carroll et al., 1990a,b; Loh and Roberts, 1990; Walsh et al., 1994). Acutely, cocaine blocks the reuptake of 5-HT (Reith et al., 1983; Ross and Renyi, 1969), and this action of the drug elevates extracellular levels of 5-HT in reward-relevant brain regions (Bradberry et al., 1993; Parsons and Justice, 1993). The cocaine-induced rise in synaptic transmitter, in turn, activates compensatory feedback mechanisms that inhibit 5-HT cell firing in the dorsal raphe (Cunningham and Lakoski, 1990; Pitts and Marwah, 1987) and suppress 5-HT biosynthesis in forebrain projection areas (Baumann et al., 1993b; Galloway, 1990). Thus, the influence of cocaine on 5-HT neurotransmission is biphasic, characterized by initial stimulation that leads to prolonged inhibition of cell activity.

Based on the acute neurochemical actions of cocaine, it might be predicted that chronic cocaine adversely affects 5-HT neuron systems. Indeed, numerous investigators have reported that prior cocaine exposure alters responsiveness to 5-HT receptor agonists in vivo (Baumann et al., 1993a; Cunningham et al., 1992; Darmani et al., 1992; Levy et al., 1992a,b, 1994b; Van de Kar et al., 1992). Interestingly, such cocaine-induced changes in 5-HT function are not accompanied by signs of overt 5-HT neurotoxicity such as reduced transmit-

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ter synthesis, transmitter depletion, or terminal degeneration (Baumann et al., 1993b; Benmansour et al., 1992; Johnson et al., 1993; Kleven et al., 1988; Paris et al., 1991; Yeh and DeSouza, 1991).

Neuroendocrine pharmacology represents a useful tool for evaluating cocaine-induced changes in 5-HT function in laboratory animals and humans (reviewed by Levy et al., 1994a). For example, the stimulatory influence of 5-HT on the hypothalamic-pituitary-adrenal axis is well documented (Fuller, 1990; Levy et al., 1994a; Van de Kar, 1991; Yatham and Steiner, 1993). Drugs that release endogenous stores of neuronal 5-HT, such as fenfluramine, elicit secretion of adrenocorticotropin (ACTH) from the pituitary and corticosterone from the adrenal cortex (Fuller and Snoddy, 1990; McElroy et al., 1984; Van de Kar et al., 1985). In addition, direct agonists that activate 5-HT_{1A} or 5-$HT_{2A/2C}$ receptors increase plasma ACTH and corticosterone (Bagdy et al., 1989; Calogero et al., 1990). Because these hormonal changes are mediated via activation of 5-HT pathways in the brain (Fuller, 1990; Levy et al., 1994a; Van de Kar, 1991), agonist-induced endocrine responses can be utilized as sensitive measures of central 5-HT function. Neuroendocrine challenge tests have been widely used in clinical studies to demonstrate changes in 5-HT responsiveness associated with depression and psychiatric disorders (see Coccaro and Kavoussi, 1994; Siever et al., 1991; Yatham and Steiner, 1993).

The purpose of the present experiments was to use a neuroendocrine approach to examine the effects of cocaine exposure on 5-HT function in rats. Following a 7-day cocaine administration period, the corticosterone responses to the 5-HT releaser fenfluramine, the 5-HT_{1A} receptor agonist 8-OH-DPAT, and the 5-$HT_{2A/2C}$ receptor agonist DOI were evaluated. All rats were fitted with indwelling jugular catheters prior to the study. 5-HT receptor agonists were administered and serial blood samples were withdrawn via the catheters. This experimental design offered several advantages: (1) rats were not subjected to handling stress at the time of 5-HT receptor agonist challenge, (2) full time-course effects of 5-HT receptor agonists could be determined in the same animal, and (3) hormonal responses could be measured in the same subjects at early (42 h) and late (8 days) phases of drug withdrawal.

2. Material and methods

2.1. Animals and surgery

Male Sprague-Dawley rats weighing 280–320 g were singly housed in standard vivarium conditions (lights on from 07:00–19:00 h) with food and water available ad libitum. Rats were anesthetized with methoxyflurane (Pittman-Moore, Phillipsburg, NJ, USA), and indwelling jugular catheters were surgically implanted (Harms and Ojeda, 1974). Each catheter (0.5 mm i.d., 1.0 mm o.d.) consisted of an 80 mm section of vinyl tubing (Dural Plastics, Auburn, N.S.W., Australia) and a 30 mm section of Silastic Medical grade tubing (Dow Corning, Midland, MI, USA) joined by an 8 mm length of 23 gauge thin-wall stainless steel tubing (Small Parts, Miami, FL, USA). The juncture of the Silastic and vinyl tubing was reinforced with 20 gauge heat-shrink Teflon tubing (Small Parts). During surgery, the Silastic end of the catheter was inserted into the jugular vein and advanced to the atrium whereas the vinyl end was exteriorized on the nape of the neck between the ears. Rats were allowed 4–5 days to recover from the surgery prior to any experimental manipulation. Catheters were flushed daily with 0.3 ml of 50 IU/ml heparin saline to maintain patency, and this routine habituated the animals to bleeding and flushing procedures. Catheters maintained in this manner typically remained patent for at least 4 weeks postsurgery.

2.2. Drugs and treatments

Cocaine and (±)-fenfluramine were obtained from the Addiction Research Center Pharmacy (Baltimore, MD, USA). The 5-HT_{1A} receptor agonist (±)-8-hydroxy-2-(di-*n*-dipropylamino)tetralin hydrobromide (8-OH-DPAT) and the 5-$HT_{2A/2C}$ receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) were purchased from Research Biochemicals (Natick, MA, USA). All drugs were dissolved in 0.9% NaCl immediately before use and injected in a volume of 1 ml/kg. Chronic treatment injections (cocaine or saline) were administered i.p., while acute challenge injections (5-HT receptor agonists or saline) were administered i.v. over 10 s intervals.

2.3. Experimental procedures

On the day of an experiment, rats were moved to the testing room in their home cages at 11:00 h and allowed 1 h to acclimate to the room surroundings. At 12:00 h, polyethylene extension tubes (PE 50; Clay Adams, Parsippany, NJ, USA) 36 cm in length were connected to the catheters and threaded outside of the cages. This arrangement enabled the investigator to perform i.v. injections and serial blood sampling without disturbing the subjects during the experiment. Catheters were flushed with 0.3 ml of 50 IU/ml heparin saline, and acute challenge injections were administered between 13:00–14:00 h.

In the first study, we examined the dose-response effects of 5-HT receptor agonists on plasma cortico-

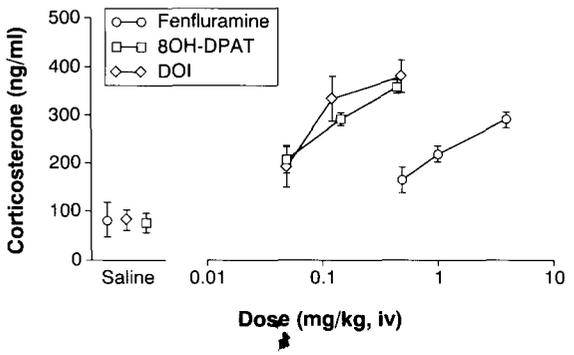

Fig. 1. Dose-response effects of intravenous administration of 8-OH-DPAT (50, 150, 450 μg/kg), DOI (50, 125, 500 μg/kg) and fenfluramine (0.5, 1, 4 mg/kg) on plasma corticosterone concentrations in male rats. The data shown are from blood samples collected 30 min after injections. Data represent mean ± S.E.M. of 7 rats per group. * $P < 0.05$ vs. saline (Duncan's test).

sterone. These initial experiments were performed to determine the optimal drug doses for use in subsequent challenge tests evaluating the effects of chronic cocaine. In this phase of the study, rats were drug-naive and tested only once. Fenfluramine (0.5, 1 and 4 mg/kg), 8-OH-DPAT (50, 150 and 450 μg/kg), DOI (50, 125 and 500 μg/kg) or saline was administered i.v., and blood samples (0.5 ml) were withdrawn immediately before, and at 30 and 60 min after injection. An equal volume of saline was infused i.v. after each blood draw to maintain volume homeostasis. Blood samples were collected into 1 ml tuberculin syringes, transferred to 1.5 ml heparinized (25 μl of 1000 IU/ml) centrifuge tubes on ice, and spun for 10 min at 5000 rpm. Plasma was decanted and stored at −80°C until the time of assay.

In the second study, we evaluated the influence of chronic cocaine administration on the corticosterone responses to 5-HT receptor agonists. Rats received cocaine (15 mg/kg i.p., b.i.d.) or saline (1 ml/kg i.p., b.i.d.) for 7 days. Animals received i.v. challenge injections of fenfluramine (1.2 mg/kg), 8-OH-DPAT (50 μg/kg), DOI (100 μg/kg) or saline at 42 h after the final chronic treatment. These drug doses were shown to produce submaximal, nearly equivalent, corticosterone responses in the dose-response study. Blood samples were collected immediately before, and at 15, 30 and 60 min after challenge injections, as described above. In this phase of the study, rats were challenged again at 8 days postchronic treatment. Essentially, the 42 h challenge experiment was repeated at 8 days so that each subject received the same 5-HT receptor agonist at an identical dose.

2.4. Corticosterone assay and data analysis

Plasma corticosterone concentrations were determined by radioimmunoassay using commercially available kits (ICN Biomedicals, Irvine, CA, USA). All samples from an experiment were analyzed in duplicate within a single assay. The average intra-assay coefficient of variability was 7.6%. Plasma corticosterone concentrations were evaluated by one-way (dose-response study) or two-way (cocaine study) analysis of variance (ANOVA). When significant F values were obtained, post-hoc comparisons were performed using Duncan's multiple range test. $P < 0.05$ was chosen as the minimum criterion for statistical significance. Analyses were performed on an IBM-compatible computer using commercially available software (BMDP Statistical Software, Los Angeles, CA, USA).

3. Results

Fig. 1 depicts the effects of 5-HT receptor agonists or saline on plasma corticosterone concentrations measured 30 min after injection. Separate groups of vehicle controls were run in parallel with each 5-HT receptor agonist, and the corticosterone concentrations 30 min after saline were similar (approximately 80 ng/ml). 8-OH-DPAT ($F(3,24) = 35.74$, $P < 0.0001$), DOI ($F(3,24) = 11.69$, $P < 0.0001$) and fenfluramine ($F(3,24) = 18.51$, $P < 0.0001$) significantly elevated plasma corticosterone in a dose-related fashion. 8-OH-DPAT and DOI exhibited similar dose-response profiles: the lowest dose of 8-OH-DPAT to significantly elevate corticosterone was 50 μg/kg, whereas the lowest effective dose of DOI was 125 μg/kg.

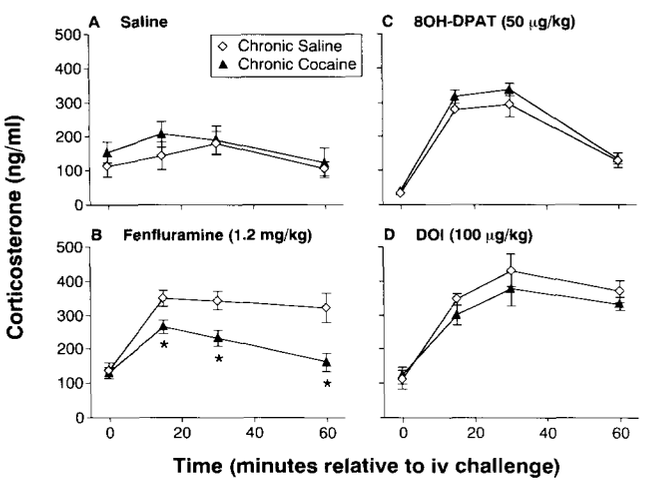

Fig. 2. Corticosterone responses to fenfluramine (1.2 mg/kg i.v.), 8-OH-DPAT (50 μg/kg i.v.) and DOI (100 μg/kg i.v.) 42 h after withdrawal from cocaine. Rats were pretreated with cocaine (15 mg/kg i.p., b.i.d.) or saline for 7 days. Blood samples were withdrawn immediately before (0 min) and at 15, 30 and 60 min after challenge injections. Data represent mean ± S.E.M. for 7–8 rats per group. * $P < 0.05$ vs. saline pretreated group (Duncan's test).

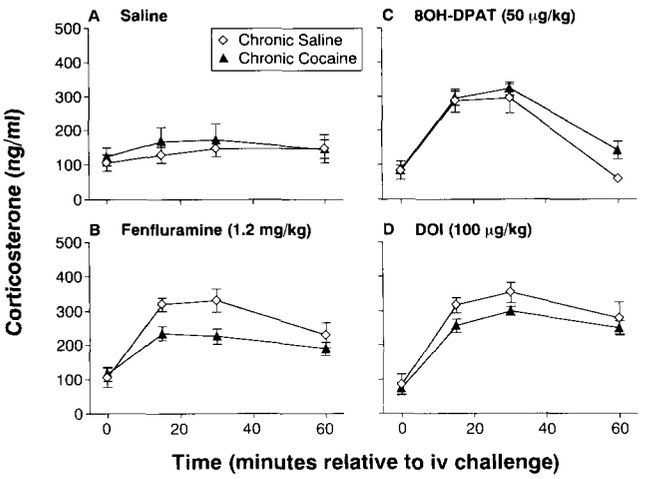

Fig. 3. Corticosterone responses to fenfluramine (1.2 mg/kg i.v.), 8-OH-DPAT (50 μg/kg i.v.) and DOI (100 μg/kg i.v.) 8 days after withdrawal from cocaine. Rats were pretreated with cocaine (15 mg/kg i.p., b.i.d.) or saline for 7 days. Blood samples were withdrawn immediately before (0 min) and at 15, 30 and 60 min after challenge injections. Data represent mean ± S.E.M. for 7–8 rats per group.

Fenfluramine was approximately 10-fold less potent than 8-OH-DPAT or DOI in its ability to increase corticosterone secretion.

The influence of cocaine exposure on the corticosterone responses to fenfluramine, 8-OH-DPAT, DOI and saline is presented in Fig. 2 and Fig. 3. Fig. 2 illustrates the responses to 5-HT receptor agonists at 42 h after the final chronic pretreatment. Basal preinjection corticosterone concentrations were approximately 100 ng/ml and did not differ between cocaine and saline groups. Regardless of pretreatment condition, acute i.v. saline challenge elevated plasma corticosterone at 15 and 30 min postinjection relative to preinjection hormone levels. However, this effect was modest and not statistically significant. The corticosterone response to fenfluramine (1.2 mg/kg i.v.) was significantly blunted in cocaine-treated rats withdrawn for 42 h ($F(1,14) = 12.12$, $P < 0.01$). Significant differences between cocaine and saline pretreated groups were observed at 15, 30 and 60 min after fenfluramine injections. In saline pretreated rats, plasma corticosterone remained elevated above baseline 60 min after fenfluramine whereas in cocaine pretreated rats hormone levels had returned to baseline at this time. In contrast, the ability of 8-OH-DPAT and DOI to increase plasma corticosterone was not affected by cocaine exposure.

The corticosterone responses to fenfluramine, 8-OH-DPAT, DOI and saline were retested at 8 days postchronic treatment. As shown in Fig. 3, the fenfluramine-induced corticosterone rise was still blunted in cocaine-treated rats when tested at 8 days after cessation of chronic treatment. However, this attenuated response to fenfluramine was no longer statistically significant ($F(1,12) = 4.35$, $P < 0.059$). The ability of 8-OH-DPAT and DOI to elevate plasma corticosterone concentrations was not affected by prior cocaine exposure.

4. Discussion

The present data suggest that repeated cocaine injections alter 5-HT neurotransmission. Corticosterone responses to the 5-HT-releasing agent fenfluramine were diminished in rats previously exposed to cocaine for 7 days. Interestingly, the actions of direct 5-HT receptor agonists stimulating 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors were not affected by prior cocaine treatment. Taken together, these observations suggest that chronic cocaine produces presynaptic functional changes in the 5-HT neurons mediating activation of the hypothalamic-pituitary-adrenal axis.

Our results confirm and extend the findings of others who have shown that repeated cocaine injections reduce the ACTH, corticosterone and prolactin responses to the 5-HT releaser, *p*-chloroamphetamine (Levy et al., 1992b; Van de Kar et al., 1992). Similarly, the ACTH response to the *d* isomer of fenfluramine is reportedly attenuated after prior cocaine exposure (Levy et al., 1994b). Levy, Van de Kar and coworkers have performed numerous endocrine challenge studies in rats to assess 5-HT function during cocaine withdrawal (reviewed by Levy et al., 1994a). It is pertinent to compare the methods used in the present work with the methods used by these investigators. In all studies (including the present one), the repeated cocaine treatment regimen was similar (15 mg/kg i.p., b.i.d., for 7 days), and injections of 5-HT receptor agonists were administered approximately 42 h after the final chronic treatment. However, in the current experiments, 5-HT receptor agonists were administered i.v. and serial blood samples were collected via indwelling jugular catheters. This differs from the work of Levy, Van de Kar and colleagues where challenge agents were administered i.p. or s.c., and trunk blood was collected after decapitation. Despite the differences in methodology, the studies are in agreement that cocaine exposure results in blunted endocrine responses to 5-HT releasers.

Fenfluramine releases endogenous stores of 5-HT by a Ca^{2+}-independent process that requires interaction with the 5-HT reuptake carrier (Berger et al., 1992; Fuller et al., 1988). It is well known that various endocrine effects of fenfluramine can be antagonized by acute pretreatment with 5-HT uptake inhibitors such as fluoxetine (Levy et al., 1994b; McElroy et al.,

1984; Van de Kar et al., 1985). McElroy et al. (1984) reported that fenfluramine-induced corticosterone secretion is blocked by fluoxetine pretreatment, but not all investigators have replicated this finding (Van de Kar et al., 1985). Data from our laboratory (Baumann, unpublished) indicate that the rise in plasma corticosterone after i.v. fenfluramine is significantly reduced by acute fluoxetine pretreatment. Thus, the corticosterone response to fenfluramine appears to reflect the activation of central 5-HT neurons.

Because cocaine is itself a potent blocker of 5-HT reuptake (Reith et al., 1983; Ross and Renyi, 1969), one possible explanation for reduced responsiveness to fenfluramine is that residual cocaine is bound to 5-HT transporters in the brain. This proposal seems unlikely in the present case for at least two reasons. First, cocaine is rapidly metabolized after injection with a half-life of 15–90 min depending upon the species and route of drug administration (Schuster, 1992). In our work, fenfluramine was administered 42 h after the last chronic treatment at a time when brain levels of cocaine would be negligible. Furthermore, we (Baumann and Rothman, unpublished) and others (Levy et al., 1994b) have shown that not all fenfluramine-mediated actions are attenuated by prior cocaine administration. For example, Levy and coworkers showed that the renin response to *d*-fenfluramine was not altered by chronic cocaine (Levy et al., 1994b). These investigators used a chronic cocaine treatment paradigm that was identical to the one used in the present work.

There are several viable mechanisms whereby cocaine could produce deficits in presynaptic 5-HT function. These possibilities include (but are not limited to) cocaine-induced alterations in 5-HT synthesis, release and/or reuptake. We have previously shown that the same cocaine injection regimen used in the present study does not alter basal 5-HT synthesis rate in microdissected regions of rat brain when examined 42 h postchronic treatment (Baumann et al., 1993b). Moreover, chronic cocaine exposure does not significantly decrease brain tissue levels of 5-HT or its acid metabolite 5-hydroxyindole acetic acid (5-HIAA) in rats (Kleven et al., 1988; Yeh and DeSouza, 1991). Parsons and Justice (1993), using microdialysis methods, found that prior cocaine treatment had no affect on basal extracellular levels of 5-HT in the nucleus accumbens or dorsal raphe of awake rats. Collectively, these findings indicate that reduced sensitivity to fenfluramine is not mediated via cocaine-induced neurotoxicity in 5-HT nerve terminals (also see Paris et al., 1991).

The available data regarding the effects of chronic cocaine on 5-HT transporter binding are less clear. Cunningham et al. (1992) reported that cocaine exposure (15 mg/kg i.p., b.i.d., 7 days) causes small, site-specific increases in the density of [^{3}H]imipramine-labeled 5-HT reuptake sites when measured 20 min after the final chronic injection. In contrast, Benmansour et al. (1992) found no changes in 5-HT transporter binding when assessed 7 days after repeated high dose cocaine treatment (35 mg/kg s.c., 10 days). While these data are not fully consistent, they indicate that reduced responsiveness to fenfluramine cannot be explained on the basis of decreased 5-HT transporter number. Recent evidence suggests that efficiency of the 5-HT reuptake mechanism can be modulated by protein phosphorylation. Anderson and Horne (1992) have shown that activators of protein kinase C decrease the V_{max} of 5-HT transport in platelets, without appreciable changes in 5-HT transporter number. Preliminary evidence suggests that brain 5-HT transporter activity can be modulated by protein kinase C activation (B.J. Hoffman, personal communication). Thus, it seems feasible that chronic cocaine exposure could alter 5-HT reuptake function without measurable changes in 5-HT transporter binding.

Based on the present data alone, we cannot fully discount the possibility that particular 5-HT receptor subtypes are rendered subsensitive by prior cocaine administration. For example, it is conceivable that changes in postsynaptic 5-HT receptor function may have gone undetected in our study because only one dose of each 5-HT receptor agonist was tested in the chronically treated animals. There is evidence that some 5-HT_{1A} receptor-mediated endocrine responses are diminished after chronic cocaine (Levy et al., 1994a,b). We failed to observe any effect of prior cocaine exposure on the corticosterone response to 5-HT_{1A} receptor activation with 8-OH-DPAT, and these results agree with the findings of Levy and colleagues (unpublished). However, these same investigators found that cocaine-treated rats exhibited a significant reduction in 8-OH-DPAT-induced ACTH secretion (Levy et al., 1994b). These observations suggest that changes in the 5-HT regulation of hypothalamic-pituitary-adrenal axis can occur without measurable changes in agonist-induced corticosterone release. Thus, plasma ACTH, rather than plasma corticosterone, is probably a more sensitive and reliable index of hypothalamic-pituitary-adrenal axis function (for discussion see Levy et al., 1994a). It should be mentioned that the reported cocaine-induced alterations in 5-HT_{1A} receptor responsiveness are not accompanied by changes in the number or affinity of 5-HT_{1A} receptor sites (Javaid et al., 1993; Johnson et al., 1993).

Data obtained in this study indicate that the cocaine-induced changes in 5-HT function exhibit a gradual return toward control levels. When retested 8 days following the final cocaine treatment, the corticosterone response to fenfluramine was still attenuated in cocaine pretreated rats. However, this blunted effect was no longer statistically significant. There have been few investigations examining the long-term changes in

5-HT function following cocaine exposure. Most studies have characterized the effects of 5-HT agents at 24–48 h postchronic treatment. Levy et al. (1993) examined the hormone responses to the 5-HT releaser *p*-chloroamphetamine in rats following 7 days of cocaine exposure. This study determined that cocaine produced long-lasting (at least 1 month) reductions in the prolactin and renin responses to *p*-chloroamphetamine, while the ACTH response approached normal values within 1 week after withdrawal from cocaine. More work will be required to accurately determine the time course of the recovery of function following cocaine exposure.

The potential clinical relevance of the present data deserves comment. Numerous studies have shown that abstinent cocaine addicts experience anhedonia, fatigue, and other mood disturbances, which resemble the symptoms of major depression (Gawin and Kleber, 1986; Weddington et al., 1991). Furthermore, it is generally accepted that the etiology of depression involves deficits in endogenous 5-HT function (see Meltzer and Lowy, 1987). In support of this proposal, several investigators have demonstrated reduced cortisol and/or prolactin responses to fenfluramine challenge in depressed patients compared to non-depressed subjects (O'Keane and Dinan, 1991; Siever et al., 1984; Weizman et al., 1988). It is tempting to speculate that cocaine-induced changes in 5-HT neurotransmission may underlie the dysphoria experienced by human addicts during acute drug abstinence. Indeed, medications which enhance central serotonergic tone have been implemented with some success in the treatment of cocaine dependence (Batki et al., 1993; Rothman et al., 1994; reviewed by Kosten, 1992).

In summary, the present results indicate that prior exposure to repeated cocaine injections causes alterations in presynaptic 5-HT function. Our data agree with numerous studies conducted in rodents that have demonstrated blunted hormone responses to 5-HT releasing agents during cocaine withdrawal (Levy et al., 1992b, 1994b; Van de Kar et al., 1992). Collectively, these findings suggest the possibility that cocaine may produce 5-HT dysfunction in humans, and neuroendocrine challenge tests should be performed to examine this hypothesis directly in cocaine addicts. Fenfluramine has been widely utilized as a probe to assess the status of 5-HT neurons in human subjects with mood and psychiatric disorders (Coccaro and Kavoussi, 1994; Siever et al., 1991). There are direct 5-HT receptor agonists such as isapirone (5-HT_{1A} receptor agonist) and *m*-chlorophenylpiperazine ($5\text{-HT}_{2A/2C}$ receptor agonist) that are available for clinical use as well (Levy et al., 1994a; Yatham and Steiner, 1993). The identification of neurobiological substrates mediating cocaine addiction and withdrawal may lead to improved strategies for treatment.

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