Brain Dopamine Transporter Messenger RNA and Binding Sites in Cocaine Users

A Postmortem Study

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Background: Results of recent radioligand binding experiments suggest that chronic cocaine exposure increases dopamine transporter (DAT) synthesis throughout the striatum of humans. However, detection of cocaine binding site increases in animals and humans has varied depending on the radioligand used. The present experiment tested the hypothesis in cocaine-using humans that synthesis of midbrain DAT messenger RNA increases parallel with increased striatal DAT binding sites.

Methods: Striatal and midbrain samples were collected during autopsy examination from human cocaine users (n = 34) and from age-, sex-, and race-matched control subjects (n = 36). Levels of DAT messenger RNA were quantified in the medial and lateral midbrain regions using in situ hybridization, and striatal DAT binding sites were assessed by quantitative autoradiography using the DAT-specific radioligand [3H]WIN 35428.

Results: Striatal DAT binding sites were markedly increased in cocaine users, but, paradoxically, medial DAT messenger RNA levels were decreased.

Conclusion: Cocaine exposure has a marked effect on DAT function, but the mechanisms involved may be complex.

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A CUTELY, COCAINE binds to the dopamine transporter (DAT) and inhibits the normal re-uptake of released dopamine from the synaptic cleft.1 Increased synaptic dopamine in the striatum and the prefrontal cortex is believed to be critical in sustaining cocaine self-administration in animals and may underlie the subjective experience of euphoria in human users.2,3 Results of recent postmortem experiments in human cocaine users show that chronic cocaine exposure leads to increased cocaine binding sites on the DAT in the striatum.4,5 This perturbation could conceivably contribute to chronic cocaine-induced clinical phenomena, including binging, withdrawal anhedonia, and craving.

Accounting for the increased striatal DAT binding detected in the previously mentioned experiments was an apparent increase in the number of binding sites. However, detection of cocaine binding site increases in humans4,7 and animals8,16 has varied depending on the radioligand used, suggesting complexity in the mechanism of regulation. The simplest cause of increased DAT binding sites would be increased synthesis of DAT molecules, resulting from increased production of DAT messenger RNA (mRNA) in midbrain dopamine cell bodies (Figure 1, D), although other mechanisms are possible (Figure 1, C). The goal of the present experiment was to test the hypothesis that midbrain DAT mRNA levels increase parallel with striatal DAT binding sites in postmortem samples from persons who used cocaine.

Brain samples were collected at autopsy from a group of chronic cocaine abusers who were also acutely using cocaine. Control subjects, not exposed to cocaine, were also selected to match the cocaine-using group based on age, sex, race, and socio-economic status (SES). As expected among a group of cocaine abusers, there was also coexisting ethanol dependence, psychosis, and mood disorders. To partially control for the use of ethanol by the cocaine users, ethanol-dependent subjects were also included as controls. After assembling the groups based on diagnostic considerations, DAT mRNA was quantitated in midbrain sections using in situ hybridization, and the results were compared in cocaine users and controls. [3H]WIN 35428 binding to striatal DAT binding sites...
SUBJECTS AND METHODS

SUBJECTS

Postmortem brain specimens were obtained at autopsy as authorized by the Office of the Wayne County Medical Examiner (Detroit, Mich), freshly dissected, and quickly frozen on dry ice and stored at −70°C until sectioned. Specimens were obtained from 34 subjects using cocaine at the time of their deaths and from 36 control subjects dying suddenly in accidents, by assault, or of cardiac causes through the same autopsy process. The Table lists demographic information and cause of death. In addition, control subjects and cocaine users were matched as well as possible by age, sex, and race, as well as SES to control for any nonspecific factors associated with impoverished nutrition and living conditions.

Cocaine-using subjects included 34 of 37 who were found in a total population of 130 subjects from whom brain tissue in the appropriate regions was available. Seven cocaine-dependent subjects were also diagnosed as opioid abusers. Controls, chosen from 93 potential controls, met the following criteria: (1) suitable method of death (rapid, not the result of a chronic condition); (2) appropriate age, sex, and race to match the cocaine-using subjects; and (3) absence of DSM-IV diagnoses other than ethanol dependence. Among the control subjects chosen to match the cocaine users were 14 of 36 meeting the criteria for an ethanol dependence disorder. Not every subject was included in both the binding experiments (involving 30 cocaine users and 34 controls) and the in situ hybridization experiments (involving 27 cocaine users and 28 controls) because the appropriate brain region was missing or slides were damaged during the assay.

CLINICAL ASSESSMENT

At least 1 knowledgeable informant was interviewed for each subject, most often a first-degree relative. Other informants included neighbors, friends, fellow workers, police officers, medical examiners, physicians, mental health personnel, and newspaper reporters. Many interviews with family members were conducted in person, and some were done by telephone. Family members provided informed consent under guidelines approved by the Institutional Review Board of the University of Michigan, Ann Arbor.

During the interview, the Family History–Research Diagnostic Criteria checklists of symptoms for mood disorders, alcoholism, and antisocial personality, anxiety disorders, and psychotic disorders were completed. The Hollingshead Inventory, an estimate of SES, was derived for every subject. Based on available evidence, DSM-IV psychiatric diagnoses were assigned at a consensus conference. When questions remained, efforts were renewed to ascertain specific information, and the diagnostic assignment was postponed. Subjects described as chronic cocaine users included 19 meeting the DSM-IV criteria for cocaine dependence, 7 meeting the criteria for cocaine abuse, and 8 who were believed to be chronic users but who lacked sufficient evidence for a definitive diagnosis to be assigned. All chronic cocaine users had positive toxicologic test results for cocaine. Subjects diagnosed as having ethanol dependence met DSM-IV criteria. The standard set for chronic ethanol use was more formal because we were able to discover considerably more information about ethanol use patterns. In 5 subjects with an ethanol dependence diagnosis, ethanol was not detected during autopsy examination. Conversely, ethanol was found in 3 cocaine-using subjects on toxicologic testing, but other evidence indicated that these subjects were not ethanol dependent. Other cocaine users who were not diagnosed as ethanol dependent may have occasionally used ethanol. All diagnoses were assigned before assays were performed.

TOXICOLOGICAL TESTING

Urine or serum samples from subjects were assayed qualitatively for the presence of cocaine, opioids, antidepressants, antipsychotic drugs, and anxiolytic drugs using a variety of methods, including radioimmunoassay, high-performance liquid chromatography, and gas chromatography–mass spectrometry. Ethanol levels were measured by dichromate microdiffusion and gas chromatography–flame ionization detection methods.

AUTORADIOGRAPHIC BINDING ASSAYS

The autoradiographic assay methods for [3H]WIN 35428 in human brain have been described previously. Briefly, 16-μm-thick sections were incubated at 0°C in TRIS buffer (50 mmol/L; pH, 7.4) with sodium chloride, 120 mmol/L, for 1½ hours. A single saturating concentration of [3H]WIN 35428 (specific activity, 3126 GBq/mmol; New England Nuclear/DuPont, Boston, Mass), 100 nmol/L, was used to assess binding in the striatal regions based on earlier work finding a high-affinity site with a Kd of 6±2 mmol/L.

was also quantified autoradiographically for comparison with DAT mRNA levels and to replicate earlier work in a new, larger, and better clinically characterized set of subjects. The relationship between [3H]WIN 35428 binding and DAT mRNA levels within individuals was examined, and a subanalysis examined the effects of comorbid ethanol abuse on brain DAT variables.

RESULTS

Cocaine users and controls were not significantly different in age (38.2±1.5 vs 38.3±1.9 years; t = 0.05; P = .96), postmortem interval (16.4±1.0 vs 17.4±0.9 hours; t = 0.75; P = .46), sex (79% vs 75% men; t = 0.43; P = .66), or race (79% vs 61% African American; t = 1.68; P = .10). However, SES was significantly different (4.3±0.2 vs 3.5±0.2; t = 2.69; P = .009).

Striatal [3H]WIN 35428 binding sites were significantly increased in cocaine users across regions (MANOVA, Wilks λ = 0.001; F1,55= 6.56). Univariate tests indicated that cocaine use increased DAT binding in controls vs cocaine users in the caudate (399±25.9 vs 569.8±33.3 kBq/mg; F1,55= 11.9; P = .001), putamen (377.4±25.9 vs 529.1±33.1 kBq/mg; F1,55= 16.0; P<.001), and accumbens (207.2±18.5 vs 299.7±37.0 kBq/mg; F1,55= 4.45; P = .04) (Figure 4).

Dopamine transporter mRNA levels were statistically different across regions in cocaine users vs

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specific binding for [3H]WIN 35428 was determined by the addition of cocaine, 30 µmol/L. After incubation, slides were washed and then exposed to film (Kodak SB-5, Kodak, Rochester, NY) for 12 to 14 days. [3H] standards ([3H] microscale standards, Amersham, Arlington Heights, Ill) were co-exposed for each cassette. Optical densities for the accumbens, caudate, and putamen were determined using an image analysis system (Microcomputer Imaging Devices, Ottawa, Ontario). Striatal topography was identified using the atlas of Tork and Hornung. 18 Optical densities were evaluated by comparison with the microscale standards and are expressed in nanocuries per milligram.

IN SITU HYBRIDIZATION

The specific in situ hybridization protocol has been described previously. 19,20 Briefly, 20-µm-thick sections were fixed in 4% paraformaldehyde solution for 1 hour, pretreated with proteinase K to facilitate probe penetration into the tissue, and acetylated with 0.25% acetic anhydride (in triethanolamine, 0.1 mmol; pH 8). The DAT riboprobe, 890-base pairs long, was transcribed in vitro using T7 (sense) and T3 (antisense) RNA polymerases, which generated [35S]-radiolabeled complementary RNA probes of high specific activity. The sections were then hybridized with the [35S]-labeled riboprobes overnight at 60°C in hybridization buffer. After hybridization, tissue sections were treated with ribonuclease to degrade any unlabeled probe, washed under stringent conditions (65°C in low salt buffer, 0.1XSSC), and dehydrated. Sections were exposed to x-ray film (X-OMAT AR, Kodak) and were developed after the appropriate exposure time (14 days). Controls included treatment of sections with ribonuclease A before hybridization, which abolished signal, and hybridization with "sense" strand complementary RNA probes, which resulted in minimal signal. Hybridization was quantified by measuring film optical densities using the image analysis system. Two slides were quantitated per subject, which was repeated in a second experiment, and the results from both experiments were averaged. Midbrain topography was identified using the atlas of van Domburg and ten Donkelaar. 21 A typical section demonstrating the areas quantified (based on Figure 5) is shown in Figure 2. Hybridization signal was quantified in the medial and lateral midbrain regions, reflecting consistent topographic and functional distinctions. 21

Results of previous experiments show that mRNA is degraded in a few postmortem brain samples for reasons that are unclear. To control for artifactually low levels of mRNA, cyclophilin mRNA was measured in contiguous sections using the previously mentioned in situ hybridization techniques and a high-specific-activity riboprobe. 19,20,24 Cyclophilin is a ubiquitous, unregulated cell structural protein unrelated to the DAT. Optical densities for DAT mRNA in each individual were divided by the cyclophilin mRNA densities derived for that individual.

To confirm the results obtained on film, slides were selected at random from 18 cocaine users and 18 paired controls, were dipped in emulsion (Kodak DBT, Kodak), and were exposed for 4 weeks. Silver grains were highly concentrated over melanin-stained cells throughout the midbrain (Figure 3). A typical ×100 magnification field was identified in the posterior medial dopamine cell groups for each subject and silver grains were counted over 20 melanin-stained cells. The coefficient of variation for grains per cell from each individual were less than 10%. The total number of cells per typical field was also recorded as a rough measure of cell density.

DATA ANALYSIS

[3H]WIN 35428 binding and DAT mRNA levels in each brain region were confirmed initially to be normally distributed (Kolmogorov-Smirnov distances calculated, all P>.10). Because the interrelationship and significance of binding or mRNA changes in different brain regions was uncertain, the general approach taken was to use an initial multivariate analysis of variance (MANOVA) followed by univariate tests to control for multiple comparisons. [3H]WIN 35428 binding and DAT mRNA levels were each initially compared in cocaine users and controls by MANOVA (cocaine effects on binding [or mRNA] in 3 [or 2] regions). [3H]WIN 35428 binding and DAT mRNA levels were further examined for correlative relationships. In addition, [3H]WIN 35428 binding and DAT mRNA were examined for relationships with alcohol diagnosis (alone and with cocaine diagnosis) by MANOVA followed by univariate tests. Other clinical data, including age, postmortem interval, sex, race, and other psychiatric diagnosis (psychosis, mood disorder, and opioid use), were examined for effect on [3H]WIN 35428 binding and DAT mRNA using MANOVA or correlational analysis, as appropriate. Initially, to confirm that cocaine users and control subjects had been well matched, age, sex, race, SES, and postmortem interval were also compared using 2-tailed t tests (without protection for multiple comparisons, which was judged more conservative). In all comparisons, α = .05.

Data are given as mean ± SEM.
result, we further analyzed the striatal binding results in cocaine users vs controls, excluding alcoholic controls, and found that the overall cocaine effect remained statistically significant (MANOVA, Wilks $\lambda = 0.02$; $F_{3,35} = 3.46$), as did the differences in each region.

No differences in DAT mRNA or $[3H]$WIN 35428 values were found in psychotic, mood-disordered, or opioid-using subjects. Age and postmortem interval were also examined for correlation with DAT mRNA and $[3H]$WIN 35428 binding parameters. No effects were found (of 14 correlations performed, none were statistically significant). There was no significant correlation between SES and $[3H]$WIN 35428 binding or mRNA hybridization signal overall or within the cocaine user or control groups. There were no differences by race or sex in DAT mRNA levels. However, African American women who were cocaine users had higher caudate DAT binding sites (African American women, controls $[n = 8]$ vs cocaine users $[n = 6]$: 384.8±59.2 vs 788.1±99.9 kBq/mg; African American men, controls $[n = 13]$ vs cocaine users $[n = 18]$: 373.7±40.7 vs 536.5±33.3 kBq/mg; white men, controls $[n = 13]$ vs cocaine users $[n = 5]$: 436.6±40.7 vs 432.9±129.5 kBq/mg); and a white woman, cocaine user $[n = 1]$: 14.8 kBq/mg). However, the effect was not significant by MANOVA (race $\times$ sex $\times$ cocaine effects on binding in 3 regions, Wilks $\lambda = 0.27$; $F_{3,35} = 1.25$), reflecting the small numbers and high variability. The apparent race $\times$ sex trend was smaller in the putamen and accumbens.

The present findings replicate earlier findings$^4$ that human cocaine users have increased $[3H]$WIN 35428 binding sites in the striatum. Staley et al$^5$ reported similar increases among 5 cocaine users who died of overdose. Our findings suggest that cocaine overdose is not critical for up-regulation of $[3H]$WIN 35428 binding sites. In another human study, although Wilson et al$^6$ did not find a statistically significant increase in $[3H]$WIN 35428 binding in 12 cocaine users, the ratio of $[3H]$WIN 35428 binding to apparent dopamine neurons (based on quantification of vesicular transporter density) was increased more than 40% in cocaine users. In contrast, Hurd and Herkenham$^7$ reported decreased postmortem $[3H]$mazindol binding in cocaine users. One in vivo single photon emission computed tomography study$^8$ found an increase in radioactive iodine ($^{123}$I) RTI-55 binding in cocaine users, whereas another did not (after several months of abstinence)$^9$.

Contradictory results of binding studies in humans and animals may be related to different radioligands’ varying degrees of overlap with DAT binding sites. Wilson et al$^{10}$ compared the effects of long-term cocaine exposure on 2 radioligand binding sites and found that striatal $[3H]$WIN 35428 binding, but not $[3H]$GBR 12978 binding, was significantly increased in rats after self-administration of cocaine for 6 weeks. Other binding studies in animals with a finding of an increase generally used $[3H]$cocaine or $[3H]$WIN 35428$^9,11$ whereas studies$^{12-15}$ with negative findings used $[3H]$GBR 12935, mazindol, or $[3H]$nomifensine—drugs structurally unique from cocaine and its congeners. These results suggest that long-term exposure to cocaine and some similar drugs may selectively alter ligand accessibility (a characteristic compatible with the adaptation shown in Figure 1, C). Other critical variables in explaining differing outcomes include cumulative cocaine dose and interval since last exposure.$^9,10$

The lack of parallel increases in DAT mRNA levels and $[3H]$WIN 35428 binding sites suggests that these 2 variables are affected by factors not closely coupled. The decrease in DAT mRNA levels is puzzling but might reflect a secondary compensation in the face of DAT protein up-regulation caused through another mechanism besides increased transcription. It is also possible that increased synthesis of DAT protein may have actually occurred through changes in message stability or translational rates.

Several previous studies$^{16,17}$ in rats reported that cocaine exposure decreases DAT mRNA levels. Xia et al$^{17}$ provided no anatomical information. Letchworth et al$^{18}$ found medial and lateral decreases in DAT mRNA, whereas Cerruti et al$^{19}$ found that DAT mRNA decreases were limited to medial dopaminergic nuclei, as in the present study. In contrast, however, both Cerruti et al and Letchworth et al reported decreases in striatal DAT binding, whereas the present experiment found increased striatal $[3H]$WIN 35428 binding.

One explanation for the decrease in DAT mRNA is loss of dopaminergic neurons. Results of other human experiments suggest that striatal dopamine terminals may be decreased in cocaine users despite increased$^{10}$ or stable$^{[3H]$WIN 35428 binding.$^{7}$ Preliminary experiments in this laboratory also found a decrease in striatal monoamine vesicular transporter binding among these same cocaine users. These results suggest that 2 opposing processes might occur in long-term cocaine users—a loss of dopamine cells or axons (perhaps resulting from long-term exposure) and a superimposed up-regulation of DAT.
function (perhaps conformational, in response to functional blockade). The present results with emulsion-dipped slides found no apparent changes in dopaminergic cells per microscopic field. However, a more sophisticated stereologic approach to cell counting is necessary to clarify this issue.

The present experiments found a small decrease in putamen binding in alcoholic controls vs nonalcoholic controls that was statistically significant by univariate analyses but not by MANOVA. Attention is drawn to this phenomenon because similar results have recently been reported in a positron emission tomography study of alcoholic controls that was statistically significant by univariate analysis but not by MANOVA. The present experiments found a small decrease in putamen binding in alcoholic controls vs nonalcoholic controls that was statistically significant by univariate analysis but not by MANOVA.

Average SES was low in controls, and even lower in cocaine users, as would be expected in a group of long-term inner-city addicts. However, no correlations were found between SES and [3H]WIN 35428 binding or mRNA levels within the cocaine user or control groups. African American women who abused cocaine seemed to have the greatest changes in caudate DAT binding sites. However, there was considerable variability among the limited number of subjects, indicating that this effect could be artifactual. Future DAT studies should take care to search for age and racial interactions with cocaine effects.

### Demographic Features of Cocaine Users and Control Subjects

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*SES indicates socioeconomic status; PMI, postmortem interval; MOD, manner of death; COD, cause of death; ACC, Asian; AA, African American; HOM, homicide; UNDT, undetermined; NAT, natural causes; ACC, accident; GSW, gunshot wound; GSW-H, gunshot wound to the head; MVA, motor vehicle accident; ASCVD, arteriosclerotic cardiovascular disease; and ellipses, data not obtained.

†Significantly different from cocaine users, Student t test, P = .009.
The present study was limited in the number of anatomical regions quantitated and posterior-anterior levels assessed, reflecting a decision to conserve tissue for examination of several variables within the same individual. Other approaches might have provided more anatomical detail. In addition, in situ hybridization using film quantitation is not the most precise method of determining changes in mRNA levels. Another limitation was that the study was unbalanced in the number of women and white subjects included. The issue of race and sex effects could have been more readily clarified with a better distribution.

Together, the preponderance of rat and human data suggest that the DAT is regulated through complex mechanisms. However, the functional significance of the various DAT states remains unclear. The short-term effects of cocaine use on DAT function are critical in causing cocaine’s powerful euphoric effects, clearly indicating the important role that dopamine uptake plays in overall reward processes. One can speculate that other, long-term perturbations in dopamine uptake may also be similarly powerful. Long-term adaptations induced by cocaine use in the DAT could contribute to prominent clinical symptoms like binging, withdrawal depression, and craving. The present results further suggest that successful therapeutic approaches for cocaine dependence may need to take into account the regulatory effects on DAT function induced by long-term cocaine exposure.

Figure 2. Photomicrograph demonstrating dopamine transporter messenger RNA distribution in human midbrain at the level the values were quantitated. MedDA indicates medial dopaminergic cells, including paranigral nucleus and posterior medial substantia nigra compacta; LatDA, lateral dopaminergic cells, including posterior lateral and posterior superior substantia nigra compacta. Nomenclature is based on the atlas of van Domburg and ten Donkelaar.21

Figure 3. Photomicrograph demonstrating hybridization of dopamine transporter riboprobe over melanin-containing cells in emulsion-dipped slides. A, Darkfield showing the location of hybridization clustered over neuronal cell bodies in a posterior medial cell group. B, Brightfield showing melanin-stained cells in the same spatial distribution as in A, identifying dopaminergic cell bodies. These photomicrographs were taken from the subject with the densest labeling to enhance reproduction of the effect.

Figure 4. Striatal [3H]WIN 35428 binding sites were increased in cocaine users vs matched control subjects in all 3 regions (unpaired t-tests between cocaine users and controls: asterisk, P < .001; dagger, P < .05). Average cocaine values increased by 43% (caudate), 40% (putamen), and 45% (accumbens). Assay was performed at a saturating concentration of [3H]WIN 35428 (100 nmol) by quantitative autoradiography.

Figure 5. Dopamine transporter (DAT) messenger RNA (mRNA) levels compared in cocaine users and matched control subjects. The results are the average of 2 independent experiments that provided similar results. To control for variable degradative effects, DAT mRNA levels were normalized by dividing by the average cyclophillin mRNA densities found in contiguous midbrain sections. Neither DAT nor cyclophillin mRNA levels were significantly different in cocaine users compared with controls before mathematical transformation. Asterisk indicates P = .02.
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