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Effects of Cocaine on Dopamine Receptor Gene Expression: A Study in the Postmortem Human Brain

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The effects of chronic cocaine exposure on dopamine D_1 and D_2 receptor gene expression in the human brain were studied in postmortem samples from chronic cocaine abusing and matched control subjects. Using in situ hybridization and receptor autoradiography to examine messenger ribonucleic acid (RNA) and binding sites, respectively, neither D_1 nor D_2 receptor expression was found to be changed in the nucleus accumbens, caudate, putamen, or substantia nigra of the cocaine-exposed subjects. Although chronic cocaine exposure can produce alterations in dopaminergic neurotransmission, sustained compensatory changes in dopamine receptor expression do not appear to occur in the human.

Key Words: Receptors, dopamine, messenger RNA, in situ hybridization, cocaine, receptor binding, human brain

Introduction

Cocaine has myriad effects on a number of neurotransmitter systems in the brain. While influencing multiple systems, however, its effects on dopaminergic neurotransmission in the limbic system appear to be a critical mechanism underlying the reinforcing properties of this drug (Kuhar et al 1991). Cocaine is able to block the dopamine transporter, which acts as a presynaptic reuptake site, thus effectively increasing intrasynaptic dopamine concentration (Ritz et al 1987; Church et al 1987; Izenwasser et al 1990). There is some evidence that there may be compensatory changes in dopamine receptor expression as well, which could be a primary effect or a secondary result of this increased intrasynaptic dopamine (Trulson and Ulissey 1987; Goeders and Kuhar 1987; Kleven et al 1990; Volkow et al 1990; Peris et al 1990; Mayfield et al 1992; Farfel et al 1992).

The mesolimbic component of the dopamine system is one of the most likely sites of action of cocaine in the brain. This system originates in the dopamine-synthesizing cells of the ventral tegmental area, located in the medial mesencephalon, which send axons rostrally to terminate in limbic regions of the brain, including the nucleus accumbens. Parallel to this system, the nigrostriatal system arises more laterally in the midbrain, sending fibers to the caudate and putamen. The nucleus accumbens as well as the caudate and putamen send fibers back to the midbrain cell groups, thus completing discrete neuroanatomical circuits associated with reinforcement and motor functions.

The specific subtypes of dopamine receptors located in these circuits appear to be complex. Although both D_1 and

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D_2 receptors have been identified in all anatomical regions associated with these systems, the recent cloning of multiple dopamine receptor subtypes in the human has revealed an additional level of neurochemical organization. The dopamine receptors cluster into two larger families: the D_1-like family contains the D_1 (Dearry et al 1990; Zhou et al 1990; Sunahara et al 1990) and D_5 (Sunahara et al 1991; Weinshank et al 1991; Grandy et al 1991) receptors, and the D_2-like family consists of the D_2 (Grandy et al 1989), D_3 (Giros et al 1990), and D_4 (Van Tol et al 1991) receptors. In addition to D_1 and D_2 receptor localization in both the motor and limbic dopamine circuits, the D_3 and D_4 receptors also appear to be expressed in these systems, with particular enrichment in the limbic circuit (Bouthenet et al 1991; Mengod et al 1992; Lévesque et al 1992). The D_1 and D_2 receptors, however, are present in overwhelming abundance compared to the other dopamine receptor subtypes.

The present study was designed to characterize the effects of chronic cocaine use on dopamine receptor binding as well as messenger RNA expression in the postmortem human brain. The focus was on the striatal and accumbens regions, which are heavily innervated by dopaminergic fibers originating in the midbrain, and contain high densities of dopamine receptors. We have initially chosen to focus on the D_1 and D_2 receptors, as they are the most abundant and best understood of the five dopamine receptors in the circuits under examination.

Methods

BRAIN ACQUISITION. Postmortem human brain specimens were obtained at autopsy from subjects suspected of chronic cocaine use, as well as from age-, gender-, and race-matched controls who were not suspected of recent substance abuse (Table 1). These autopsies were authorized and performed by the Office of the Chief Medical Examiner, State of North Carolina, who found no evidence of neurological disease. Two blocks of brain tissue were obtained at autopsy: a midbrain slice containing the substantia nigra and ventral tegmental area, and a rostral forebrain block containing the caudate, putamen, and nucleus accumbens. These two blocks were frozen on powdered dry ice, and stored at −80°C. Nine pairs of midbrain blocks were obtained for this study, but only six pairs of the forebrain blocks were available.

CLINICAL CHARACTERIZATION. A research psychiatrist and a psychologist identified and interviewed informants for each subject. In all cases, an attempt was made to identify family members who could provide historical information. Informants included family members, neighbors, co-workers, police officers, physicians, medical examiners, and media reporters. Interviews were conducted to obtain information to determine if subjects met DSM-III-R criteria (American Psychiatric Association 1987) for affective, anxiety, psychotic, and/or psychoactive substance abuse disorders. After collection of these clinical data, DSM-III-R diagnoses were assigned at a consensus conference involving experienced research psychiatrists. For some subjects, insufficient evidence was obtained to determine if criteria were met for a formal DSM-III-R cocaine-related diagnosis; nevertheless, in these cases, subjects were clearly established to be chronic cocaine users. The presence or absence of cocaine was confirmed in urine, liver, or serum samples from all subjects by thin layer chromatography.

TISSUE PREPARATION. Blocks that were obtained at the time of autopsy remained at −80°C until the time of study. Each block was slowly warmed to −20°C and mounted for cryostat sectioning. Sections were cut 20 μm thick, and were thaw-mounted onto poly-L-lysine subbed 50 × 75 mm microscope slides. Sections were dried and stored at −80°C until further processing.

IN SITU HYBRIDIZATION. Sections were removed from −80°C storage and placed in 4% formaldehyde at 4°C. Slides were fixed for 60 min, and then processed for in situ hybridization as previously described (Meador-Woodruff et al 1989; Mansour et al 1990; Meador-Woodruff et al 1991). Slides were hybridized with $[^{35}S]$-UTP labeled riboprobes in 75% formamide hybridization buffer at 55°C overnight. Following hybridization, slides were treated with RNase A, and washed under progressively more stringent conditions, culminating with a 60 min wash in 0.5 × SSC (300 mmol/L NaCl, 30 mmol/L sodium citrate, pH 7.2) at 55°C. The slides were then dehydrated in graded alcohols, air-dried, and apposed to Kodak XAR-5 film for 1–3 weeks.

A riboprobe to the human D_1 receptor probe was synthesized using SP6 RNA polymerase, from a 396 base pair (bp) fragment that was subcloned into pGEM-3Z. This insert is a *Bgl* II-*Hind* III fragment of a genomic clone for the human D_1 receptor (bases 208-603), which encodes the region of the D_1 receptor between transmembrane domains II and V and containing the second intracytosolic loop. The D_2 receptor probe was synthesized using T7 RNA polymerase from a 446 bp fragment subcloned into pGEM-4. This insert contains the region encoding the third intracytosolic loop and transmembrane domains VI and VII of this receptor, and corresponds to bases 1044–1489 of a human D_2 receptor cDNA. This D_2 probe equally recognizes both the short and long isoforms of D_2 receptor messenger ribonucleic acid (mRNA).

A series of technical control studies was performed to

ensure the specificity of each riboprobe used in this study, as we have previously described (Meador-Woodruff et al 1989; Mansour et al 1990; Meador-Woodruff et al 1991). Both "sense-strand" and RNase-pretreated "antisense"-labeled sections were run in parallel with "antisense"-labeled sections. Under the stringent conditions used in this study, specific hybridization was observed only in the "antisense"-labeled condition.

RECEPTOR AUTORADIOGRAPHY. Preliminary studies were performed on slide-mounted human brain sections to determine the affinities of [^{3}H]raclopride (69.5 Ci/mmol, New England Nuclear) and [^{3}H]SCH23390 (85.6 Ci/mmol, New England Nuclear), selective D_2 and D_1 receptor ligands, respectively. Repeated saturation studies using concentrations of 0.75–10 nmol/L of tritiated ligand suggested that [^{3}H]raclopride had a mean K_d of 1.62 nmol/L and [^{3}H]SCH23390 (in the presence of 1 umol/L ketanserin) had an affinity of 3.17 nmol/L in human brain tissue. These values were valuable in choosing the ligand concentrations in normal and cocaine-exposed brains. Three ligand concentrations were used; one at the K_d value, a second at 1/10the K_d and a third high concentration three times the K_d. For [^{3}H]raclopride, these corresponded to 0.17, 1.55 and 4.09 nmol/L. For [^{3}H]SCH23390, the concentrations were 0.28, 2.97, 9.10 nmol/L. All incubations with [^{3}H]SCH23390 also contained 1 μmol/L ketanserin to block any binding to $5HT_2$ receptors. Nonspecific D_2 binding was defined by 1 μmol/L (+)butaclamol while nonspecific D_1 binding was evaluated with 1 μM SCH23390. Although three concentrations are not normally sufficient to determine K_d and B_{max} values, the limited availability of tissue made a more modified procedure necessary.

Prior to incubation with the tritiated ligands all slides were preincubated 15 min (22°C) in a 50 mmol/L Tris buffer (pH 7.4), containing 120 mmol/L NaCl, 5 mmol/L KCl, and 1 mmol/L $MgCl_2$. Slides were then dried and 2 ml of [^{3}H] ligand and buffer (50 mmol/L Tris (pH 7.4), 120 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.1% ascorbic acid) at the three concentrations indicated earlier was applied to the brain sections. Adjacent sections were used to evaluate nonspecific binding at each ligand concentration. Incubations were terminated at 90 min (22°C) by four 2-min ([^{3}H]raclopride) or 4 min ([^{3}H]SCH23390) Tris washes (50 mmol/L pH 7.0, 4°C) containing 120 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$ and 0.1% ascorbic acid. Slides were then dipped into distilled water (4°C), dried with a hair dryer set to cool, and apposed to tritium sensitive Hyperfilm (Amersham) with calibrated radioactive standards.

DATA ANALYSIS. Films from in situ hybridization and receptor autoradiography experiments were digitized and optical densities in anatomical regions of interest were evaluated using quantitative densitometry. Regions of interest for this study included the nucleus accumbens, caudate, and putamen for all riboprobes and radioligands; the pars compacta of the substantia nigra for D_2 receptor mRNA and binding; and the pars reticulata of the substantia nigra for D_1 receptor binding. Although the ventral tegmental area was identifiable in the midbrain blocks with a riboprobe to tyrosine hydroxylase mRNA, the probes used in this study did not generate a signal that was sufficient to convincingly quantify.

For in situ hybridization films, mean optical density values were obtained for each region studied for each probe from 8–10 slides per subject. These values were averaged to provide a single value for each region and each probe for each subject. For quantification of receptor binding, the optical density for each region of interest was converted to fmoles of ligand bound using calibrated tritium standards. Nonspecific binding was subtracted at each ligand concentration in each anatomical structure, and the values were fitted to Scatchard plots using the LIGAND program (Munson and Rodbard 1980). The K_d and B_{max} obtained from these data points agreed well with previous results using a larger series of concentrations. In all cases, mean values were compared by paired two-tailed t-tests, with $p < 0.05$ selected to define significant differences.

Results

CLINCIAL ASSESSMENT. As shown in Table 1, cocaine-abusing and control subjects were well matched for age, gender, race, and postmortem interval. There were no significant differences between the two groups for any of these items. All of the cocaine-abusing subjects were determined to be chronic cocaine users, and all had positive toxicology for cocaine at the time of death. None of the control subjects had evidence for a substance abuse or significant axis I psychiatric diagnosis, and all were free of cocaine by toxicological analysis at the time of death.

DOPAMINE RECEPTOR EXPRESSION. As shown in Figure 1, there were no significant differences between the cocaine-abusing and control groups for dopamine D_1 or D_2 receptor mRNA or binding in any of the three forebrain structures studied. Similarly, as demonstrated in Figure 2, D_2 receptor binding and mRNA as well as D_1 receptor binding were not significantly different between these groups in the substantia nigra. The ventral tegmental area was poorly visualizable in these subjects. A comparison was made of the medial versus lateral portions of the cells that were identifiable in the midbrain, however; there were neither medial-lateral differences within either subject group, nor were there differences between the groups when com-

Table 1. Clinical and Demographic Characteristics of Subjects

Cocaine subjects				Control subjects			
Age	Race/gender	PMI	Mode of death	Age	Race/gender	PMI	Mode of death
28	B/F	20	stabbing	30	B/F	14	cardiac
24	W/M	9	stabbing	27	W/M	8	stabbing
32	W/M	18	cardiac	24	W/M	12	gsw
29	W/M	8	gsw	24	W/M	16	? cardiac
37	B/F	9	overdose (cocaine)	33	B/F	9	? cardiac
21	B/F	7	gsw	29	B/F	10	stabbing
36	B/M	5	overdose (cocaine)	20	B/M	16	mva
39	B/M	7	overdose (cocaine)	41	B/M	16	gsw
36	W/F	8	cardiac	25	W/F	11	gsw

PMI: postmortem interval (hours); gsw: gunshot wound; mva: motor vehicle accident.

paring just the medial or lateral aspects of the substantia nigra (data not shown).

Discussion

Dopamine D_1 and D_2 receptor expression were not significantly different between cocaine-abusing subjects and controls in any of the three forebrain dopaminoiceptive regions studied, or in midbrain dopamine-containing cells.

Previous reports on the effects of cocaine on brain D_2 receptor expression in rats have found different effects in the nucleus accumbens versus the striatum. Immediately following chronic cocaine treatment in the rat, D_2 receptor binding has been reported consistently to be elevated in the nucleus accumbens (Trulson and Ulissey 1987; Goeders and Kuhar 1987; Kleven et al 1990; Peris et al 1990). Conversely, results in the striatum have been variable, being reported as both elevated (Trulson and Ulissey 1987) and decreased (Goeders and Kuhar 1987; Kleven et al 1990). In most studies finding an increase in the accumbens, D_2 receptor binding had returned to normal levels a week after discontinuation of cocaine (Kleven et al 1990; Peris et al 1990).

Several reports of D_1 receptor binding following chronic cocaine treatment in the rat have also been published. In one, both striatal and accumbens D_1 receptor binding were found to be decreased immediately following treatment, with the accumbens but not the striatal levels normalizing within a week or two (Kleven et al 1990). Another study, however, found no changes in either striatal or accumbens D_1 receptor binding following a week of cocaine treatment (Mayfield et al 1992).

Because species differences may complicate comparison with the current human findings, primate studies are of special note. In particular, the size, location, and possible functional significance of the nucleus accumbens differ in primates versus rodents. One study has examined dopamine and other neurotransmitter systems in the rhesus monkey following chronic cocaine exposure (Farfel et al 1992). In contrast to the rat, D_2 receptor binding in the accumbens, caudate, and substantia nigra was unchanged. A significant decrease in D_1 binding in the caudate was found, however, with no effect in the accumbens. Similarly, although not statistically significant, there is a trend toward decreased levels of D_1 receptor mRNA in forebrain structures in the present data. D_1 receptor binding, however, showed equivalent levels in cocaine-using and control subjects, suggesting that translational compensation may have occurred in the face of an alteration in gene transcription.

The effect of cocaine on D_2 receptor binding in the human has also been studied in vivo using positron emission tomography (PET) (Volkow et al 1990). Cocaine-dependent subjects who were withdrawn from cocaine in the week prior to study exhibited a decrease in apparent D_2 receptor binding (as determined by [^{18}F] N-methylspiroperidol uptake) in the striatum. In contrast, cocaine-dependent subjects who had been abstinent for 3 weeks were not distinct compared to controls. In our data, there is no suggestion of a D_2 receptor alteration in any forebrain structure. It is somewhat difficult to reconcile these findings, but may well be related to any number of technical factors, including the use of different radioligands, kinetic assumptions made in the PET study, differing limits of resolution of the two techniques, and differences in what is actually measured in each study (i.e., striatal uptake of the tracer in the PET study versus binding of radioligand to brain tissue in the present work). As Volkow and associates indicate in their report, the use of N-methylspiroperidol in PET to estimate D_2 receptor availability is an area of controversy.

A critical limitation in comparing results from animal experiments to the present data is the potential difference in frequency of administration of cocaine. In these past rodent studies, cocaine was administered at regularly scheduled intervals related to drug half-life, presumably

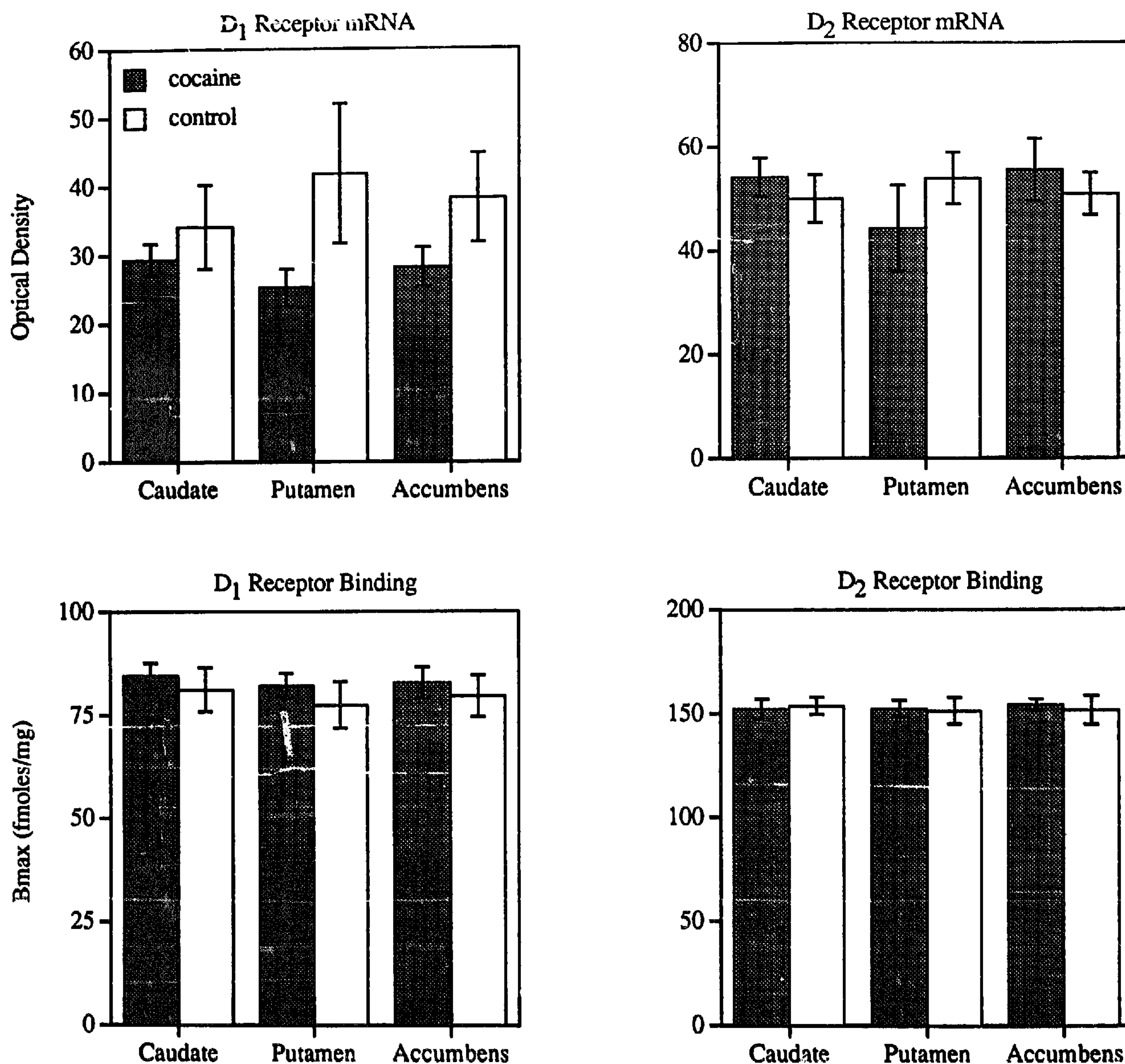

Figure 1. Dopamine receptor binding and mRNA in forebrain structures. Dopamine D_1 and D_2 receptor binding were determined with [^{3}H]SCH23390 and [^{3}H]raclopride, respectively, and D_1 and D_2 receptor mRNAs with [^{35}S]-labeled riboprobes. Results are means ± SEM for $n = 6$ subjects per group. None of the differences between control and cocaine-abusing subjects are significant.

to maintain sustained brain levels of this drug throughout the experiment. In the cocaine-abusing human, however, it is unreasonable to expect that the drug will be ingested on a regular basis for extended periods due to a number of factors, including access and availability of the drug. A more likely pattern of cocaine use in the cocaine-dependent human subject is binging, which could be described as intense but intermittent exposure. None of the animal studies reviewed above included self-administration, which may best model the human intake pattern. It appears that these two receptors are extremely plastic during and after cocaine treatment, as demonstrated by the rapid normalization of their expression following cocaine withdrawal in these rat studies. The negative findings presented in the current work represent dopamine receptor status at one uncontrolled time point along a dynamic continuum, and the absence of apparent regulation may not be altogether unexpected.

In contrast, cocaine exposure appears to increase binding to the presynaptically located dopamine transporter.

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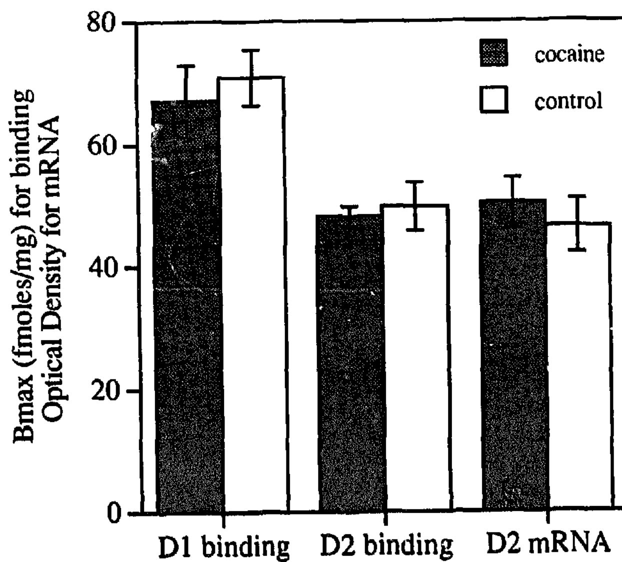

Figure 2. Dopamine receptor binding and mRNA in the substantia nigra. Dopamine D_1 and D_2 receptor binding were determined with [^{3}H]SCH23390 and [^{3}H]raclopride, and D_2 receptor mRNA with a [^{35}S]-labeled riboprobe. Results are presented as means ± SEM with n = 9 subjects per group. None of the differences between control and cocaine-abusing subjects are significant.

withdrawal in these rat studies. The negative findings presented in the current work represent dopamine receptor status at one uncontrolled time point along a dynamic continuum, and the absence of apparent regulation may not be altogether unexpected.

In contrast, cocaine exposure appears to increase binding to the presynaptically located dopamine transporter. Striatal tissue from many of these same subjects included in the present study was found to demonstrate a significant increase in [^{3}H]WIN35428 (a cocaine congener) binding (Little et al 1992). In addition to suggesting that the dopamine transporter is regulated by cocaine in the human, these particular data indicate that the lack of regulation of the D_1 and D_2 receptors in our present work was not caused by the lack of chronic cocaine exposure in the subjects.

There are several limitations to this study that need to be acknowledged. By its very design, a postmortem study is retrospective, especially in terms of diagnosis and assessment. Although the chronicity of use of cocaine is unquestioned in this subject group, it is unfortunately reconstructed from informants. The relatively small number of subjects available may also be a factor in explaining the lack of statistical findings, but with most group means as close as they were, this is unlikely. Postmortem studies inherently study a subject at a single point in time, lacking the ability to follow a subject, or the regulation of a system, in a longitudinal fashion. Despite these limitations, however, at the present time, postmortem analysis appears to be the only method that is available to image specific gene products (i.e., receptor mRNA) in the human brain.

By examining the mRNAs encoding these dopamine receptors, the present study provides an additional level of neurochemical information that has not been previously available. The simultaneous examination of both binding sites and mRNA provides distinct but complementary information. By examining both mRNA as well as receptor binding, the products of transcription and translation can be both indirectly accessed. In addition, the combined use of these data provides insight into discrete neuroanatomical circuits. D_2 receptor binding in the midbrain is likely all local, and reflects receptors functioning as autoreceptors in the midbrain. D_2 receptor mRNA in the midbrain encodes receptors that remain in the midbrain, presumably serving as autoreceptors, as well as encoding autoreceptors that exist in terminals in the forebrain. D_1 receptor binding in the midbrain is encoded in the forebrain, which is expressed as receptors on terminals in the pars reticulata of the substantia nigra. As all of this D_1 receptor binding is encoded by mRNA in cells located more rostrally, no D_1 receptor mRNA is seen in the mesencephalon; rather, the mRNA encoding midbrain D_1 receptors is located in the forebrain. D_1 and D_2 receptor mRNA in the forebrain structures is all associated with intrinsic cells in the regions of interest. D_2 receptor binding likely is on cell processes of both intrinsic cells, as well as on terminals from neurons projecting from other regions, such as the cortex; additionally, some D_2 binding reflects autoreceptors that are encoded in the substantia nigra, and is thus associated with some of the D_2 receptor mRNA visualized in the nigra. D_1 receptor binding in the forebrain is in large part on cellular processes from intrinsic cells in the forebrain structures. As can be seen, this circuitry is complex, and these data provide information concerning the regulation (or lack thereof) of multiple functional and anatomical levels of neural organization.

Other subtypes of dopamine receptors exist, especially in the limbic system. Although the D_5 receptor is not located in the limbic or motor dopamine systems (Tiberi et al 1991; Meador-Woodruff et al 1992), D_3 and D_4 receptors are present in the limbic system (Bouthenet et al 1991; Van Tol et al 1991; Mengod et al 1992; Lévesque et al 1992), although at low levels of expression. It may be interesting to determine if one or the other of these putatively more "limbic" receptors is influenced by chronic cocaine in the human. This may be difficult at this time, however, due to the low abundance of mRNAs encoding these receptors, as well as due to the lack of a D_4-specific ligand for receptor autoradiography.

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