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Dopamine transport function is elevated in cocaine users

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Abstract

Dopaminergic transmission has been suggested to be a primary mechanism mediating reinforcement, withdrawal and craving associated with psychostimulant addiction. Pyschostimulants attenuate dopamine transporter (DAT) clearance efficiency, resulting in a net increase in synaptic dopamine levels. Re-uptake rate is determined by the number of functional DAT molecules at the membrane surface. Previous *in vivo* imaging studies in humans and *in vitro* studies in post-mortem human brain have demonstrated that chronic cocaine abuse results in a neuroadaptive increase in DAT-binding site density in the limbic striatum. Whether this increase in DAT availability represents an increase in the functional activity of the transporter is unknown. Here, we present evidence that DAT function is elevated by chronic cocaine abuse. The effect of increasing post-mortem interval on the functional viability of synaptosomes was modeled in the baboon brain. Baboon brains sampled under conditions similar to human brain autopsies yielded synaptosomal preparations that were viable up to 24 h post-mortem. Dopamine (DA) uptake was elevated twofold in the ventral striatum from cocaine users as compared to age-matched drug-free control subjects. The levels of [^{3}H]DA uptake were not elevated in victims of excited cocaine delirium, who experienced paranoia and marked agitation prior to death. In keeping with the increase in DAT function, [^{3}H]WIN 35,428 binding was increased in the cocaine users, but not in the victims of excited delirium. These results demonstrate that DA uptake function assayed in cryopreserved human brain synaptosomes is a suitable approach for testing hypotheses of the mechanisms underlying human brain disorders and for studying the actions of addictive drugs in man.

Keywords: binding, cocaine, dopamine transporter (DAT), delirium, post-mortem, uptake.
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The mesolimbic dopaminergic system is an important pathway mediating reinforcement and addiction to drugs of abuse (Self and Nestler 1995). Dopaminergic neurotransmission is terminated by its re-uptake through a sodium- and chloride-dependent dopamine transporter (DAT; Krueger 1990; McElvain and Schenk 1992). The DAT is a target for cocaine and other psychostimulants (Ritz *et al.* 1987; Amara and Kuhar 1993). Cocaine potentiates dopaminergic neurotransmission by blocking the re-uptake of dopamine (DA), leading to marked elevations in the synaptic level of the neurotransmitter (for review see Giros and Caron 1993). Because cocaine binds to the DAT, regulatory changes in the dopaminergic system may occur within the brains of cocaine addicts. Alterations in DA transport function may be one of the mechanisms by which cocaine use progresses to compulsive dependence.

Regulation of DAT-binding site densities has been studied *in vitro* in the post-mortem brain of cocaine addicts and *in vivo* in acutely abstinent cocaine-dependent individuals. The cocaine congeners [^{3}H]WIN 35,428 and [^{125}I]RTI-55 (β-CIT) have been used to visualize the regional density of DAT-binding sites in the human brain from cocaine users that came to autopsy (Staley *et al*. 1994, 1995; Little *et al*. 1999). Some of the previous studies (Little *et al*. 1993, 1999; Staley *et al*. 1994, 1995), but not all (Hurd and Herkanham 1993; Wilson *et al*. 1996) have reported increased numbers of DAT-binding sites using radiolabeled cocaine congeners. While the precise explanation for the conflicting results is unclear, the use of a non-cocaine-like monoamine uptake blocker for measurements (Hurd and Herkenham 1993) or the coexisting loss of DA nerve terminals in severely dependent cocaine users (Wilson *et al*. 1996) may explain the differences (for a review see Mash and Staley 1996; Little *et al*. 1999). In keeping with the increased density of

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Abbreviations used: DAT, dopamine transporter; ED, excited delirium; SPECT, single-photon emission computed tomography.

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DAT-binding sites in human cocaine users, similar changes have been demonstrated recently in cocaine self-administering rhesus monkeys (Letchworth *et al*. 2001). A progression of changes in DAT-binding site density was demonstrated as a result of escalating dose and increasing duration of cocaine self-administration. The elevation in DAT densities in cocaine self-administering monkeys helps to rule out the potential confounds of co-morbid psychiatric conditions, different patterns and severity of abuse, and possible genotypic variations inherent in human studies. While initial cocaine exposure in primates resulted in a significant decrease in DAT-binding sites, longer exposures of 3 months to 1 year resulted in elevated densities within the limbic sectors of the striatum (Letchworth *et al*. 2001), suggesting that the altered number of DAT-binding sites is a state marker of cocaine dependence.

In vivo single-photon emission computed tomography (SPECT) measurements of DAT densities in living cocaine users also vary depending on the time since the last cocaine administration. For example, when the DAT was imaged *in vivo* within 96 h of drug abstinence, [^{123}I]]β-CIT uptake in the striatum was approximately 20% higher in the cocaine users compared to age-matched drug-free control subjects (Malison *et al*. 1995, 1998). After a prolonged period of drug abstinence (3–18 months), [^{123}I]β-CIT measures were still elevated but decreased in their level of statistical significance and demonstrated a trend toward a return to baseline values measured in drug-free control subjects (Malison *et al*. 1998). Furthermore, at 2 weeks' abstinence and beyond there was an increasing positive correlation between plasma HVA and striatal [^{123}I]β-CIT uptake (Bowers *et al*. 1998). Chronic cocaine users had significantly lower [^{11}C]cocaine uptake in the basal ganglia and thalamus when imaged at 10–90 days after the last cocaine use (Volkow *et al*. 1992). The uptake of [^{11}C]cocaine was negatively correlated with cocaine craving and with depressive symptoms, suggesting an association between withdrawal symptoms and DAT densities.

The results of human and primate studies suggest that the DAT up-regulates in response to repeated cocaine use, but may gradually normalize or decrease with long periods of drug abstinence. This compensatory increase in DAT numbers may result in a net decrease in the synaptic levels of DA. In human subjects, the magnitude of the self-reported high following cocaine administration is correlated with the degree of DAT occupancy (Volkow *et al.* 1997). If the regulatory change in DAT number is associated with an increase in transport function, it may help to explain the addictive liability of cocaine, as the change from the baseline DAergic state would be greater in cocaine-dependent individuals. The present study was undertaken to test whether cocaine exposure leads to regulatory changes in DA transport function. The uptake of [^{3}H]DA was measured in metabolically active synaptsomes isolated from cryoprotected human brain. The number of [^{3}H]WIN35,428-binding sites on the DAT was quantified in parallel to [^{3}H]DA uptake.

Materials and methods

Materials

All drugs and chemicals were obtained from Sigma Chemical (St Louis, MO, USA). [^{3}H]DA (48 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, USA). [^{3}H]WIN35,428 (84.5 Ci/mmol) was purchased from New England Nuclear (Dupont, Boston, MA, USA).

Neuropathological tissue specimens

Post-mortem neuropathological specimens were obtained during routine autopsy from age-matched drug-free control subjects. Medicolegal investigations of the deaths were conducted by forensic pathologists. Neuropathological examinations were done on all subjects and demonstrated that the cases included in this study were free of any gross or histopathological changes in brain. The circumstances of death and toxicology data were reviewed carefully before classifying a cocaine intoxication case with or without preterminal excited delirium (ED). Fatal ED victims exhibited an acute onset of bizarre and violent behavior, which was characterized by one or more of the following: aggression, combativeness, hyperactivity, extreme paranoia, demonstration of unexpected strength, or incoherent shouting (Wetli and Fishbain 1985; Wetli *et al*. 1996). The syndrome of fatal ED is defined as accidental cocaine toxicity in subjects who exhibited bizarre and violent behavior (as described above) followed by fatal cardiorespiratory arrest (Ruttenber *et al*. 1997). All cases were evaluated for common drugs of abuse and alcohol, and positive urine screens were confirmed by quantitative analysis of blood. Blood cocaine was quantified using gas-liquid chromatography with a nitrogen detector. Frozen brain regions were sampled for quantitation of cocaine and benzoylecognine using gas chromatography/mass spectroscopy techniques as described previously (Hernandez *et al*. 1994). Drug-free age-matched control subjects were selected from accidental or cardiac sudden deaths with negative urine screens for all common drugs and there was no history of licit or illicit drug use prior to death.

Cryopreservation of post-mortem human and baboon brains

Since it is virtually impossible to rapidly obtain autopsy tissue from human brain immediately after death or to confirm that a case is HIV-negative within a time frame of less than 8 h, it is not feasible to conduct measures of DA uptake on the same day that the brains come to post-mortem examination. We have utilized a procedure taken from previous studies (Hardy *et al*. 1983; Dodd *et al*. 1986) for cryopreserving brain tissue in a way that allows assessments of functional DA uptake. Briefly, specific regions-of-interest were dissected, diced and placed into pre-weighed polyethylene bags (Kapack^{TM}, 3M, Minneapolis, MN, USA) containing 10 mL ice-cold 0.32 M sucrose and sealed. These were then placed into a thick-walled (~2 cm) polystyrene box containing cotton wool for insulation and the lid was sealed. The entire box was then transferred into a freezer set at −70°C for slow freezing for at least 24 h before use.

To determine the effects of post-mortem interval on the parameters of uptake (K_m and V_{max} values), Baboon brains (*Papio*

hamadryas) were taken at routine sacrifice ($n = 24$). Brains were left *in situ* at 4°C for extended time periods to duplicate the storage conditions of human cadavers. After removal of the brain from the calvarium, regions-of-interest were dissected into small blocks and slowly frozen in ice-cold 0.32 M sucrose as described above.

DA uptake assay

Measurement of DA uptake was performed on human and baboon synaptosomes as described (Eshleman *et al*. 2001). Briefly, blocks of cryopreserved human ventromedial striatum were placed into 10–15 mL sucrose (0.32 M) and slow-thawed at 25°C, then removed from sucrose, blotted dry and weighed. Sucrose (0.32 M, 10 vol per original wet weight of tissue, 4°C) was added and tissue was homogenized by hand with 10–20 strokes of a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C, and the supernatant was decanted and centrifuged at 20 000 *g* for 20 min at 4°C. The pellet was resuspended in 10 volumes of glucose (0.32 M)/original wet weight of tissue at 4°C. For baboon synaptosomes, fresh and cryopreserved striata were prepared as described for human brain. Krebs–HEPES buffer (pH 7.4, 800 μL) was placed in a 25°C water bath, synaptosomes (100 μL) were added, and the assay was initiated by the addition of [^{3}H]DA (20 nM final concentration). For estimates of K_i values, synaptosomes were pre-incubated with competitor drug at 25°C for 10 min. Specific uptake was defined as the difference in uptake observed in the absence and presence of either mazindol (5 μM) or (–)cocaine (100 μM). To obtain the estimates of the affinity (K_m) and maximal velocity of transport (V_{max}) of [^{3}H]DA, the specific activity of [^{3}H]DA was decreased by increasing concentrations of unlabeled DA. Uptake was terminated after 5 min by filtration through Whatman 934-AH filters (Clifton, NJ, USA) pre-soaked in 0.1% polyethylenimine. Each assay was conducted with duplicate determinations for the baboon and triplicate for human brain assays.

[^{3}H]WIN35,428 binding to the DAT

Saturation binding of [^{3}H]WIN35,428 in human brain was conducted as described previously (Staley *et al*. 1994). Briefly, the ventral striatum was dissected from slowly frozen and fast-thawed brain specimens that were cryoprotected in sucrose for pair-wise comparisons with DA uptake. The brain tissue was homogenized in 20 volumes of 10 mM sodium phosphate buffer (pH 7.4) containing 0.32 M sucrose using a Brinkman polytron and centrifuged at 32 000 *g* for 15 min. The membrane pellet was washed one time, and resuspended in ice-cold sucrose–phosphate buffer at a final dilution of (1 : 20; w/v). A fixed concentration of [^{3}H]WIN35,428 (0.5 nM) was incubated with human membranes (5 mg/mL) in the presence of increasing concentrations of unlabeled WIN35,428 (0.1 nM – 10 μM) for 2 h at 4°C. The binding reaction was stopped by rapid filtration through 934AH filters pre-soaked in 0.1% polyethyleneimine. The filters were washed with ice-cold buffer (3 × 4 mL) with radioactivity measured by liquid scintillation spectrometry. Proteins were determined by the method of Lowry *et al*. (1951).

Data analysis

Estimated K_m and V_{max} values for [^{3}H]DA uptake and K_d and B_{max} values for [^{3}H]WIN35,428-binding data were analyzed using non-linear least-squares curve-fitting program LIGAND and GraphPad PRISM (v3.0; GraphPad, San Diego, CA, USA). Simultaneous statistical analysis of a one-site versus two-site model were made using LIGAND in which the two-site model was preferred only when the *F*-test displayed significance at the $p < 0.01$ level. Correlation coefficients were calculated using the non-parametric method of Spearman, with significance set at $p < 0.05$. Group differences were assessed by one-way ANOVA and *post hoc* analyses were conducted using the Dunnett's test and results were considered statistically significant when $p < 0.05$.

Results

Cocaine-related fatalities were identified and classified as part of an ongoing case–control study of the toxicology reports, scene descriptions, supplemental background information, and autopsy findings (Escobedo *et al*. 1991; Ruttenber *et al*. 1997). The cocaine users were selected for the present study based on evidence of a number of surrogate variables of chronicity, including the review of prior arrest records, hospital and substance abuse treatment admission, and from structured interviews with next-of-kin or other informants at the time of death. Toxiciological and epidemiological data reported previously by our group demonstrates that a pattern of cocaine use characterized by repeated binges is associated with the development of fatal ED (Ruttenber *et al*. 1997). The cocaine users ($n = 10$; eight male, two female), ED victims ($n = 8$; seven male, one female) and control subjects ($n = 10$; seven male, three female) were not significantly different in demographic characteristics. Their mean ages were 38.3 ± 3.5, 35.3 ± 3.5 and 37.8 ± 2.7 years, respectively. The post-mortem intervals did not differ significantly across groups (cocaine users = 14.2 ± 1.0; ED = 12.1 ± 1.5; control subjects 14.7 ± 1.7). Excited cocaine delirium cases have been included in this study as a comparison group. This psychiatric syndrome is comprised of delirium with marked agitation, respiratory depression, and sudden death (Wetli *et al*. 1996; Ruttenber *et al*. 1997). The mode of death and agonal state are important variables when investigating neurotransmitter function in human brain (Wester *et al.* 1985). The cause of death for cocaine users was cocaine intoxication ($n = 8$) and cocaine-related homicides ($n = 2$). The cause of death for the control subjects was cardiac sudden death for eight of the cases. Two control cases were homicide victims. Thus, there was no confound of protracted agonal state, as all of the cocaine users, ED victims and control subjects died suddenly.

Cocaine and benzoylecognine (BE) were detected in urine and blood at the time of death for all of the cocaine users. Two of the cocaine cases and one of the ED victims had alcohol detected in post-mortem blood at low levels (BAC < 0.01%). The concentration of cocaine and its principal metabolite BE were measured in blood samples obtained at autopsy. The average (mean ± SEM) blood levels of cocaine and BE were 6.7 ± 5.5 and 3.5 ± 1.3 mg/L,

respectively, in the cocaine users. The ED victims exhibited 10-fold lower levels of cocaine (0.4 ± 0.3 mg/L), but comparable levels of BE (2.3 ± 1.1 mg/L) in blood. None of the control cases tested positive for any neuroactive drug or metabolite. None of the cases selected for this study tested positive for opiates in blood and there was no opiate or opiate metabolite measured in urine toxicology screens.

DA uptake

In preliminary experiments, we optimized the assay conditions for [^{3}H]DA uptake in baboon and human striatal synaptosomes. Specific [^{3}H]DA uptake by synaptosomes prepared from the ventromedial striatum was linear for up to 10 min (Fig. 1). Based on these data and the results in human striatal synaptsomes (data not shown), all assays were conducted utilizing a 5-min incubation at 25°C. Uptake of [^{3}H]DA increased linearly from 20 to 200 μg protein per assay. For all subsequent assays, 125 ± 8 μg protein (from an original wet weight of approximately 10 mg resuspended in 10 volumes of 0.32 M glucose) were used for all assays. Maximal uptake rates for DA were determined in both human and baboon striatal preparations.

The effect of freezing on DA uptake in striatal synaptosomes is illustrated in Fig. 2. Cryopreserved baboon brain specimens had significantly lower [^{3}H]DA uptake as compared to freshly prepared striatal synaptosomes ($t = 2.8$; $p < 0.05$). The results of the analysis of Eadee–Hofstee plots of the data gave estimates of K_m and V_{max} values in the baboon and human striatum that were in good agreement (Fig. 3). Freezing of the brain in isotonic sucrose had no effect on the K_m for DA uptake in baboon brain (data not shown). Two experiments were conducted on fresh human

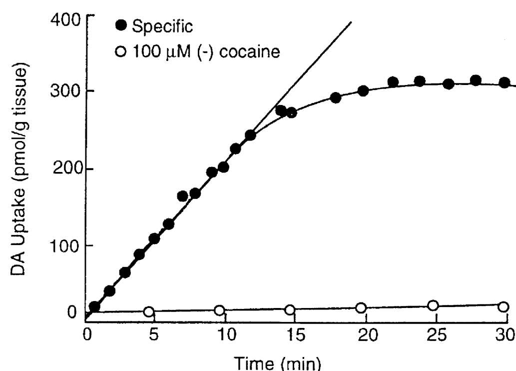

Fig. 1 Time-course of [^{3}H]DA uptake in cryopreserved baboon striatal synaptosomes. Uptake of [^{3}H]DA (20 nM) uptake was assessed over time (0–30 min) in baboon (*Papio hamadryas*) synaptosomes (10 mg tissue original wet weight). Non-specific uptake was determined using 5 μM mazindol. The data shown are the results of the mean of three independent experiments each conducted in duplicate (post-mortem interval = 2 h). The straight line represents the results of least squares linear regression of the data between t = 0–10 min. ●, Specific; ○, 100 μM (-) cocaine.

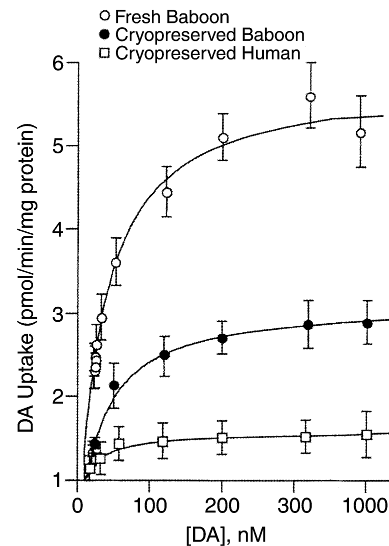

Fig. 2 [^{3}H]DA uptake by synaptosomes prepared from non-frozen (○) and cryopreserved baboon (●) striatum and from cryopreserved human (□) caudate. Data shown are the mean ± SEM of four experiments conducted in duplicate. The V_{max} and K_m values are given in the Results section.

brain preparations. The results in freshly prepared human synaptosomes demonstrated that there was no effect of freezing on the K_m for DA (411.9 nM vs. 406 ± 41 nM) or the K_i for cocaine (252.7 nM vs. 197 ± 41), respectively.

The effect of increasing post-mortem intervals on DA uptake in baboon striatum is shown in Fig. 4. The K_m for DA was not altered by increasing post-mortem interval ($r = 0.23$, $n = 9$, $p = 0.56$). The average K_m value for DA uptake was 130.8 ± 22.9 nM. Correlational analysis demonstrated that post-mortem interval had a significant effect on the V_{max} for DA ($r = 0.87$, $n = 9$, $p = 0.002$). The V_{max} for uptake of [^{3}H]DA into synaptosomes was decreased over the first 8 h, after which a stable plateau was reached and maintained up to 24 h (Fig. 4). For DA uptake assays, striatal tissues were taken from specimens with post-mortem intervals that ranged from 12 to 18 h in order to provide reliable estimates of kinetic parameters across individual cocaine cases and control subjects.

Regulation of DA uptake by cocaine use

The initial rates of DA uptake into synaptosomes from cocaine users, ED victims and age-matched drug-free control subjects is shown in Fig. 5. Initial rates of DA uptake were measured over a range of concentrations (20–2000 nM). Analysis of the data revealed high-affinity DA uptake across all subjects. A series of DA uptake curves analyzed by Eidee–Hofstee plots were best fit to a single component of uptake when either 100 μM cocaine or 5 μM mazindol was used to define specific uptake (Fig. 5). The affinities for DA uptake in cocaine users (377 ± 45 nM) was not significantly

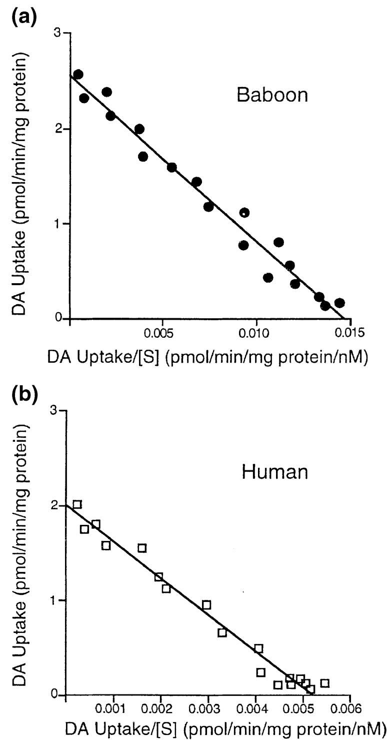

Fig. 3 Saturation analysis of [^{3}H]DA uptake in baboon and human striatum. [^{3}H]DA was present at 20 nM and increasing concentrations of unlabeled DA were added up to 10 000 nM. Non-specific uptake was defined with 100 μM cocaine. The straight line represents the best fit chosen by the LIGAND program. Shown is a typical experiment from (a) baboon brain (post-mortem interval 12 h) and (b) human control subject (White male, age 32, post-mortem interval = 12 h). The baboon striatum gave a K_m value = 130.2 nM, and a V_{max} = 2.56 pmol/min/mg protein, r^2 = 0.982. Data from a representative experiment performed on cryopreserved human ventral striatum gave a K_m value of 408 nM and a V_{max} of 1.98 pmol/min/mg protein, r^2 = 0.971.

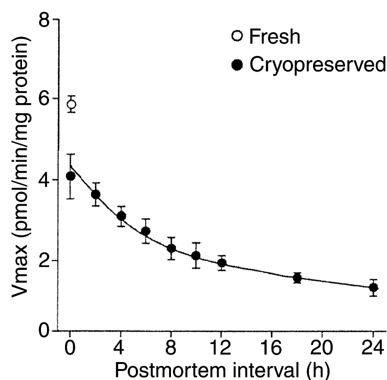

Fig. 4 The effect of post-mortem interval on [^{3}H]dopamine uptake. Striatal synaptosomes were prepared from baboon brain tissue specimens which were left *in situ* in the calvarium until the time points indicated. V_{max} values were taken from linear transformation of the saturation analysis of [^{3}H]DA uptake at each time point. Data shown are the result of the mean of three independent experiments with error bars (SEM), each conducted in triplicate. ○, Fresh; ●, cryopreserved.

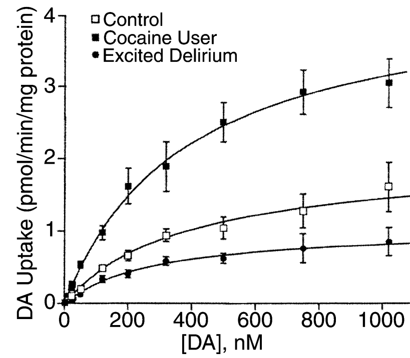

Fig. 5 Dopamine transport function in control subjects (□), cocaine users (■) and victims of excited cocaine delirium (●). Dopamine uptake versus substrate plots in ventral striatal synaptosomes from control subjects (n = 10), cocaine users (n= 10), and excited delirium victims (n = 8). Data are means ± SEM of the individual experiment each performed in triplicate.

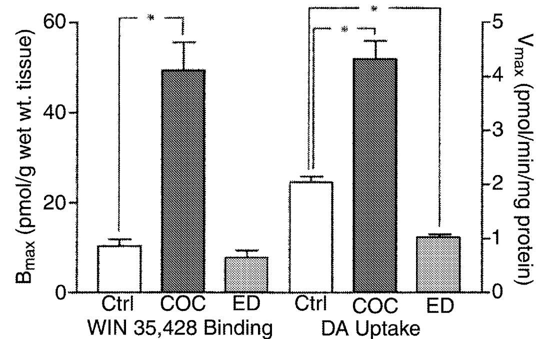

Fig. 6 Striatal [^{3}H]WIN35,428 binding and [^{3}H]DA uptake in control subjects, cocaine users and victims of excited cocaine delirium. Bars shown on the left represent B_{max} values for high-affinity WIN35,428 binding sites. Results shown on the right are V_{max} values for DA uptake. Student's t-test was used to compare cocaine users and excited delirium victims to control subjects and cocaine users to excited delirium victims. *$p < 0.01$.

different from control subjects (406 ± 41). DA transport affinity was lower in the excited delirium group (303 ± 41), but the difference was not statistically significant as compared to either control subjects ($p > 0.05$) or cocaine users ($p > 0.05$). The maximal uptake rate for DA was elevated significantly in cocaine users compared to control subjects ($p < 0.01$; Fig. 6). In contrast, the average V_{max} value for the ED group was significantly decreased from age-matched drug-free control subjects ($p < 0.01$). These results demonstrate that dopamine transport function was elevated twofold in cocaine users. Victims of fatal ED failed to demonstrate an increase in DA transport function, even though these cases had long-standing histories of chronic drug use and they came to post-mortem evaluation with cocaine and cocaine metabolites measured in blood at the time of death.

Regulation of DAT levels

The [^{3}H]WIN35,428 saturation experiments resulted in a series of curves from the 28 cases that were assayed in parallel with measures of DA uptake. In agreement with previous results (Staley *et al*. 1994), Rosenthal transformation of the independent determinations in each group revealed curvilinear plots suggesting two binding components (data not shown). When the data were fit to either a one- or a two-component model, the two-component model was the statistically preferred fit (F-value range, 10.55–42.9; $p < 0.01$). The average K_d values for the high-affinity sites was 3.8 ± 0.4 nM for the control subjects, 4.9 ± 0.7 nM in the cocaine users and 3.4 ± 0.3 in the ED subjects. The dissociation constants reported here for all three groups were not statistically different and are in very good agreement with previous estimates of the high-affinity component reported in the primate (Madras *et al*. 1989) and human striatum (Little *et al*. 1993, 1999; Pristupa *et al*. 1994; Staley *et al*. 1994).

The densities corresponding to the high-affinity binding component were elevated significantly in the cocaine users as compared with age-matched drug-free control subjects ($p < 0.01$; Fig. 6). The dissociation constants and densities corresponding to the low-affinity component of [^{3}H]WIN35,428 binding were unchanged by cocaine exposure (range 62.4–71.8 nM; data not shown). Victims of excited cocaine delirium failed to show an increase in [^{3}H]WIN35,428 binding and had B_{max} values for the high-affinity sites that were comparable to the control subjects (Fig. 6). These results demonstrate that cocaine use results in an increase in the density of the high-affinity component of [^{3}H]WIN35,428-binding in the human brain. However, the number of DAT-binding sites in ED victims were not significantly lower compared to cocaine users ($p > 0.05$). The lack of an up-regulation in DAT levels was in keeping with the lower maximal rates of DA uptake measured in this subgroup of cocaine users.

Discussion

We have investigated the effect of cocaine abuse on DA transport in the human brain post-mortem. These findings provide the first demonstration of adaptations in DA uptake in post-mortem human brain. In cocaine users, DAT levels and DA uptake were elevated in the ventral striatum compared to age-matched drug-free control subjects. ED victims exhibit profound neuropsychiatric complications prior to death. Victims of excited cocaine delirium failed to exhibit an up-regulation of DAT function or levels, suggesting a different dynamic of DA turnover in response to repeated blockade of re-uptake in this subgroup of cocaine users. The regulatory profile shown here provides additional support for the role of the DAT in cocaine dependence.

While a number of studies have provided important information on the regulation of DAT-binding sites in primate (Letchworth *et al*. 2001) and human brain post-mortem (Hurd and Herkenham 1993; Little *et al*. 1993, 1999; Staley *et al*. 1994; Wilson *et al*. 1996), there is a lack of data regarding DA uptake in human brain. Previous studies have shown that viable brain tissue preparations can be obtained from human brain at autopsy (for review see Dodd *et al*. 1986). The development of validated methods for studying DA uptake in post-mortem human brain samples affords the opportunity for correlating functional changes in DA uptake with radioligand binding to the DAT.

Cryopreserved brain specimens that have undergone a slow-freeze and fast-thaw gave K_m values for DA that were very similar to freshly prepared synaptosomes. Although the substrate affinities and potencies of inhibitors of DA uptake are well correlated in cryopreserved human brain, rat brain and recombinant hDAT (Eshleman *et al*. 2001), the maximal uptake rates were lower in cryopreserved human and baboon brain as compared to fresh tissue. We have optimized procedures to obtain active preparations of normal and cocaine exposed brain tissue specimens. In contrast to metabolic activity, the majority of CNS receptors and transport binding sites and many second messenger systems are known to be stable over a very long post-mortem delay time (Hardy *et al*. 1983; Dodd *et al*. 1986). We conducted studies in baboon brain to determine the effect of post-mortem interval on DA transport function. The results shown here in baboon brain are in agreement with a previous study of the effects of autolysis on rat striatal synaptosomes (Haberland and Hetey 1987). Thus, cryopreserved brain tissues can afford optimal tissue integrity and consistent measures of DA uptake when sampled within a tightly matched range of post-mortem intervals.

DAT-binding sites have been reported to increase in cocaine self-administering rhesus monkeys (Letchworth *et al*. 2001) and human cocaine addicts (Little *et al*. 1993, 1998; Staley *et al*. 1994; Malison *et al*. 1998; but see Hurd and Herkenham 1993; Wilson *et al*. 1996). To some extent, controversy about the regulation of DA transporter sites in human brain may be due to a lack of rigor in measurement and examination of cocaine exposure. It is possible that the effects of cocaine on the DAT may only occur at higher doses over long periods of use. We have compared cocaine users that came to autopsy with documented histories of highest patterns of cocaine use to individuals with no exposure. Although every attempt is made to document the pre-mortem pattern of cocaine use (amount, duration, and total lifetime use), it is more difficult to collect absolute exposure measures from interviews with informants and next-of-kin. The recent study by Letchworth *et al*. (2001) demonstrates unequivocally that chronic cocaine exposure results in an up-regulation in DAT-binding sites in the striatum, that was dependent on dose and length of cocaine exposure. The elevation in DAT-binding sites had a topographic pattern that was marked over the ventromedial caudate, putamen,

and nucleus accumbens. This topography is very similar to the regulatory pattern reported previously for *in vitro* [^{3}H]WIN35,428 binding to the DAT in human cocaine users (Staley *et al*. 1994). The present study extends and confirms these previous findings, as striatal DA uptake is increased in parallel to [^{3}H]WIN35,428-binding in human cocaine users who had long-term histories of abuse.

The development of therapeutic agents that block the binding of cocaine and other abused drugs, but not DA transport function, is one possible strategy for treating cocaine dependence (Rothman 1990; Johnson and Vocci 1993; Vocci *et al*. 1995; Kuhar *et al*. 1999). Several different lines of evidence suggest that it may be possible to block cocaine binding to the DAT but not affect basal DA uptake. Chimeras constructed of portions of the DAT and the norepinephrine transporter suggest that different domains of the protein are important for determining the affinity of substrates and inhibitors and maximal transport rates (Giros *et al*. 1994; Buck and Amara 1995). Single nucleotide substitutions in the DAT cause differential effects on substrate uptake and cocaine-analog binding (Uhl *et al.* 1998; Lin *et al.* 2000). Vaughan (1995) demonstrated protection of different fragments of the DAT from proteolytic digestion by structurally dissimilar DAT uptake inhibitors. These results suggest that structurally dissimilar DA uptake inhibitors and substrates bind to overlapping, but non-identical sites on the DAT.

The identity of specific binding sites on the native DAT have been characterized with a number of different radioligands, including [^{3}H]cocaine, and the cocaine congeners [^{3}H]WIN35,428, [^{125}I]RTI-55 and [^{125}I]RTI-121 (for review see Mash and Staley 1996). These cocaine congeners label multiple sites with a pharmacological profile characteristic of the DAT in rat, primate, and human striatum (Madras *et al*. 1989; Boja *et al*. 1992; Izenwasser *et al.* 1993; Staley *et al*. 1994; Gracz and Madras 1995; Allard *et al*. 1996). Controversy exists over the existence and relevance of high- and low-affinity binding sites in both rat and human brain (Wilson *et al*. 1996; Reith and Selmeci 1992; for review see Izenwasser 1998). While there appears to be a correlation between two binding sites (Boja *et al*. 1992; Izenwasser *et al*. 1993), and two components for DA uptake (Izenwasser *et al*. 1992, 1993) in rat, the relationship of these binding and uptake components to cocaine-induced behaviors is not clear. However, several studies have suggested that the high-affinity cocaine-binding site is related to the locomotor activating (Cline *et al*. 1992a; Izenwasser *et al*. 1992; Katz *et al*. 1996) and discriminative stimulus (Cline *et al*. 1992b) effects of cocaine. The relevance of high- and low-affinity binding sites for the cloned and native human DAT to the functional state of the carrier protein is unknown (Pristupa *et al*. 1994). We have shown previously a differential regulation by cocaine of high- and low-affinity [^{3}H]WIN35,428-binding sites on the human DAT (Staley *et al*. 1994). The results of the present study confirm that the high affinity [^{3}H]WIN35,428-binding site is up-regulated by cocaine. This may reflect a post-translational modification of the native protein that alters the efficiency of the re-uptake process. Although the pharmacological identity of cocaine recognition sites and their relevance to DA transport function has remained unclear, the results shown here demonstrate that up-regulated DA transport occurs in parallel with a change in high affinity [^{3}H]WIN35,428-binding sites.

The clinical effects of cocaine intoxication are characterized by extreme agitation, irritability or affective lability, impaired judgment, paranoia, hallucinations (visual or tactile), and sometimes manic excitement. Medical and psychiatric symptoms caused by acute cocaine intoxication are a common reason for presentation to the emergency department. A case series of cocaine abuses who died following a syndrome of excited cocaine delirium was first described in 1985 (Wetli *et al*. 1996). We have previously examined the regulatory patterns of DAT in victims of excited delirium using ligand binding and *in vitro* autoradiography. Autoradiographic mapping with a single concentration of [^{3}H]WIN35,428 and [^{125}I]RTI-55 failed to demonstrate an elevation in the apparent density of the DA transporter in the striatum of ED subjects as compared to drug-free age-matched control subjects (Staley *et al*. 1995). In the present study, Rosenthal analysis of saturation binding curves for [^{3}H]WIN35,428 demonstrated a significant decrease in total binding sites for the excited delirium subgroup of cocaine overdose victims. Analysis of curvilinear Rosenthal plots demonstrated that there was no change in the apparent density of the high-affinity cocaine recognition site in the ED victims as compared to age-matched drug-free control subjects. However, the density of the low-affinity cocaine recognition site was significantly decreased in the ED victims (Staley *et al*. 1995). The pathological mechanism accounting for the lower DAT numbers in this subgroup of cocaine users is not currently understood. The decrease in DAT numbers as compared to other chronic cocaine users may reflect toxicity to pre-synaptic DAergic neurons. Lower DAT densities have been observed with abuse of other psychostimulants such as methamphetamine that target the DAT (McCann *et al*. 1998; Villemagne *et al*. 1998). The lack of an up-regulation of DAT may contribute to the development of a persisting hyperdopaminergic state in this pathological disorder. The lack of an up-regulation of DAT binding and transport function in victims of excited delirium may result in elevated extracellular levels of DA with repeated cocaine use. This failed up-regulation of DA transport and the accompanying persisting elevations in extracellular DA may explain the development of excited delirium.

The use of cocaine is associated with acute increases in DA levels in the synaptic cleft and overstimulation of post-synaptic receptors. Although DAT densities are lower in the

ventral compared to dorsal striatum, elevations in extracellular DA were found to be greatest in the ventral striatum of baboons after acute, non-contingent amphetamine use (Drevits *et al*. 1999). In keeping with these findings, Bradberry (2000) observed progressive increases in extracellular DA concentrations in the ventromedial and central striatum, but not the dorsolateral striatum after 6 months of 'recreational' cocaine use (one drug-taking session per week). These results are in keeping with the findings in monkeys (Letchworth *et al*. 2001) and human cocaine users (Staley *et al*. 1994), that the largest increases were observed in portions of the striatum that have lower DAT binding levels. Chronic stimulation of the DAT may lead to a new adaptive state in the CNS and alter the dynamics of DA turnover as a response to repeated blockade of DA re-uptake. Based on the results shown here, it is likely that chronic cocaine use, by causing an increase in DAT levels in the pre-synaptic membrane, would result in a decrease in the amount of DA available to stimulate post-synaptic receptors. The need to maintain homeostasis within central DAergic systems may be one of the factors that drives the compulsive use of cocaine.

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References

Allard P., Marcusson J. O. and Ross S. B. (1996) [^{3}H]WIN35,428 binding in the human brain. *Brain Res.* **706**, 347–350.

Amara S. G. and Kuhar M. J. (1993) Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* **16**, 73–93.

Boja J. W., McNeill R. M., Lewin A. H., Abraham P., Carroll F. I. and Kuhar M. J. (1992) Selective dopamine transporter inhibition by cocaine analogs. *Neuroreport* **3**, 984–986.

Bowers M. B., Malison R. T., Seibyl J. P. and Kosten T. R. (1998) Plasma homovanillic acid and dopamine transporter during cocaine withdrawal. *Biol. Psychiatry* **15**, 278–281.

Bradberry C. W. (2000) Acute and chronic dopamine dynamics in a nonhuman primate model of recreational cocaine use. *J. Neurosci.* **20**, 7109–7115.

Buck K. J. and Amara S. G. (1995) Structural domains of catecholamine transporter chimeras involved in selective inhibition by antidepressants and psychomotor stimulants. *Mol. Pharmacol.* **48**, 1030–1037.

Cline E. J., Scheffel U., Boja J. W., Carroll F. I., Katz J. L. and Kuhar M. J. (1992a) Behavioral effects of novel cocaine analogs: a comparison with *in vivo* receptor binding potency. *J. Pharmacol. Exp. Ther.* **260**, 1174–1179.

Cline E. J., Terry P., Carrol F. I., Kuhar M. J. and Katz J. L. (1992b) Stimulus generalization from cocaine to analogs with high *in vitro* affinity for dopamine uptake sites. *Behav. Pharmacol.* **3**, 113–116.

Dodd P. R., Hardy J. A., Baig E. B., Kidd A. M., Bird E. D., Watson W. E. J. and Johnson G. A. R. (1986) Optimization of freezing, storage, and thawing conditions for the preparation of metabolically active synaptosomes from frozen rat and human brain. *Neurochem. Pathol.* **4**, 177–198.

Drevits W. C., Price J. C., Kupfer D. J., Kinahan P. E., Lopresti B., Holt D. and Mathis C. (1999) PET measures of amphetamine-induced release in ventral versus dorsal striatum. *Neuropsychopharmacology* **221**, 694–709.

Escobedo L. G., Ruttenber A. J., Agocs M. M., Anda R. F. and Wetli C. V. (1991) Emerging pattterns of cocaine use and the epidemic of cocaine overdose deaths in Dade County, Florida. *Arch. Pathol. Lab. Med.* **115**, 900–905.

Eshleman A., Wolfron K., Mash D. C., Christensen K. and Janowsky A. (2001) Drug interactions with the dopamine transporter in cryopreserved human caudate. *J. Pharm. Exp. Ther.* **296**, 442–449.

Giros B. and Caron M. G. (1993) Molecular characterization of the dopamine transporter. *Trends Pharmacol. Sci.* **14**, 43–49.

Giros B., Wang Y. M., Suter S., McLeskey S. B., Pifl C. and Caron M. G. (1994) Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J. Biol. Chem.* **269**, 15985–15988.

Gracz L. M. and Madras B. K. (1995) [^{3}H]WIN 35,428 ([^{3}H]CFT) binds to multiple charge-states of the solubilized dopamine transporter in primate striatum. *J. Pharmacol. Exp. Ther.* **273**, 1224–1234.

Haberland N. and Hetey L. (1987) Studies in post-mortem dopamine uptake 1. Kinetic characterization of the synaptosomal dopamine uptake in rat and human brain after post-mortem storage and cryopreservation. Comparison with noradrenaline and serotonin uptake. *J. Neural. Transm.* **68**, 289–901.

Hardy J. A., Dodd P. R., Oakley A. E., Perry R. H., Edwardson J. A. and Kidd A. M. (1983) Metabolically active synaptosomes can be prepared from frozen rat and human brain. *J. Neurochem.* **40**, 608–614.

Hernandez A., Andollo W. and Hearn W. L. (1994) Analysis of cocaine and metabolites in brain using solid phase extraction and full-scanning and GC/ion trap mass spectrometry. *Forensic Sci. Int.* **65**, 149–156.

Hurd Y. L. and Herkanham M. (1993) Molecular alterations in the neostriatum of human cocaine addicts. *Synapse* **13**, 357–369.

Izenwasser S. (1998) Basic pharmacological mechanism of cocaine. In: *Cocaine Abuse: Behavior, Pharmacology and Clinical Applications* (ed. J. Katz), pp. 1–20. Academic Press, New York.

Izenwasser S., Rosenberger J. G. and Cox B. M. (1992) Inhibition of [^{3}H]dopamine and [^{3}H]serotonin uptake by cocaine. comparison between chopped tissue slices and synaptosomes. *Life Sci.* **50**, 541–547.

Izenwasser S., Rosenberger J. G. and Cox B. M. (1993) The cocaine analog WIN 35,428 binds to two sites in fresh rat caudate-putamen: significance of assay procedures. *Life Sci./Pharmacol. Lett.* **52**, 141–145.

Johnson D. N. and Vocci F. J. (1993) Medications development at the National Institute on Drug Abuse: focus on cocaine. *NIDA. Res. Monogr.* **135**, 57–70.

Katz J. L., Newman A. H. and Izenwasser S. (1996) Relations between heterogeneity of dopamine transporter binding and function and the behavioral pharmacology of cocaine. *Pharmacol. Biochem. Behav.* **57**, 505–512.

Krueger B. K. (1990) Kinetics and block of dopamine uptake in synaptosomes from rat caudate nucleus. *J. Neurochem.* **55**, 260–267.

Kuhar M. J., McGirr K. M., Hunter R. G., Lambert P. D., Garrett B. E. and Carroll F. I. (1999) Studies of selected phenyltropanes at monoamine transporters. *Drug Alcohol Depend.* **56**, 9–15.

Letchworth S. R., Nader M., Smith H., Friedman D. P. and Porrino L. J. (2001) Progression of changes in dopamine transporter binding site density as a result of cocaine self-administration in rhesus monkeys. *J. Neurosci.* **21**, 2799–2807.

Lin Z., Masanari I. and Uhl G. R. (2000) Dopamine transporter proline mutations influence dopamine uptake, cocaine analog recognition and expression. *FASEB J.* **14**, 716–728.

Little K. Y., Kirkman J. A., Carroll F. I., Clark T. B. and Duncan G. E. (1993) Cocaine use increases [^{3}H]WIN 35,428 binding sites in human striatum. *Brain Res.* **628**, 17–25.

Little K. Y., McLaughlin D. P., Zhang L., McFinton P. R., Dalack G. W., Cook E. H. J., Cassin B. J. and Watson S. J. (1998) Brain dopamine transporter messenger RNA and binding sites in cocaine users: a postmortem study. *Arch. Gen. Psychiatry* **55**, 793–799.

Little K. Y., Zhang L., McLaughlin D. P., Desmond T., Frey K. A., Dalack G. W. and Cassin B. J. (1999) Striatal dopaminergic abnormalities in human cocaine users. *Am. J. Psychiatry* **156**, 238–245.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. A. (1951) Protein measurement with the Folin phenol reagent. *J.Biol. Chem.* **193**, 265–275.

McCann U. D., Wong D. F., Yokoi F., Villemagne V., Dannals R. F. and Ricaurte G. A. (1998) Reduced striatal dopamine transporter density in abstinent methamphetamine and methcathinione users: evidence from positron emission tomography studies with [^{11}C]WIN-35,428. *J. Neurosci.* **18**, 8417–8422.

McElvain J. S. and Schenk J. O. (1992) A multisubstrate mechanism of striatal dopamine uptake and its inhibition by cocaine. *Biochem. Pharmacol.* **43**, 2189–2199.

Madras B. K., Spealman R. D., Fahey M. A., Neumeyer J. L., Saha J. K. and Milius R. A. (1989) Cocaine receptors labeled by [^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl) tropane. *Mol. Pharmacol.* **36**, 518–524.

Malison R. T., Best S. E., Wallace E. A., McCance E., Laruelle M., Zogbhi S. S., Baldwin R. M., Seibyl J. S., Hoffer P. B., Price L. H., Kosten T. R. and Innis R. B. (1995) Euphorigenic doses of cocaine reduce [^{123}I]β-CIT SPECT measures of dopamine transporter availability in human cocaine addicts. *Psychopharmacology* **122**, 358–362.

Malison R. T., Best S. E., van Dyck C. H., McC., Ance E. F., Wallace E. A., Laruelle M., Baldwin R. M., Seibyl J. P., Price L. H., Kosten T. R. and Innis R. B. (1998) Elevated striatal dopamine transporters during acute cocaine abstinence as measured by [^{123}I]β-CIT SPECT. *Am. J. Psychiatry* **155**, 832–834.

Mash D. C. and Staley J. K. (1996) Cocaine recognition sites on the human dopamine transporter in drug overdose victims. In: *Neurotransmitter Transporters. Structure and Function* (M. E. A. Reith., ed.), pp. 56–67. Humana, New York.

Pristupa Z. B., Wilson J. M., Hoffman B. J., Kish S. J. and Niznik H. B. (1994) Pharmacological heterogeneity of the cloned and native human dopamine transporter. Dissociation of [^{3}H]WIN 35,428 and [^{3}H]GBR 12935 binding. *Mol. Pharmacol.* **45**, 125–135.

Reith M. E. A. and Selmeci G. (1992) Radiolabeling of dopamine uptake sites in mouse striatum-comparision of bindings-sites for cocaine, mazindol, and GBR 12935. *Nauym-Schmeidebergs's Arch. Pharmacol.* **345**, 309–318.

Ritz M. C., Lamb R. J., Goldberg S. R. and Kuhar M. J. (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* **237**, 1219–1223.

Rothman R. B. (1990) High affinity dopamine reuptake inhibitors as potential cocaine antagonists: a strategy for drug development. *Life Sci.* **46**, L17–L21.

Ruttenber A. J., Lawler-Heavner J., Yin M., Wetli C. V., Hearn W. L. and Mash D. C. (1997) Fatal excited delirium following cocaine use: Epidemiologic findings provide new evidence for mechanisms of cocaine toxicity. *J. Anal Toxicol.* **42**, 25–31.

Self D. W. and Nestler E. J. (1995) Relapse to drug-seeking. neural and molecular mechanisms. *Drug Alcohol Depend.* **51**, 49–60.

Staley J. K., Hearn W. L., Ruttenber A. J., Wetli C. V. and Mash D. C. (1994) High-affinity cocaine recognition sites on the dopamine transporter are elevated in fatal cocaine overdose victims. *J. Pharmacol. Exp. Ther.* **271**, 1678–1685.

Staley J. K., Wetli C. V., Ruttenber A. J., Hearn W. L. and Mash D. C. (1995) Altered dopaminergic synaptic markers in cocaine psychosis and sudden death. *NIDA Res. Monogr. Series* **153**, 491.

Uhl G., Lin Z., Metzger T. and Dar D. E. (1998) Dopamine transporter mutants, small molecules, and approaches to cocaine antagonist/ dopamine transporter disinhibitor development. *Methods Enzymol.* **296**, 456–465.

Vaughan R. A. (1995) Photoaffinity-labeled ligand binding domains on dopamine transporters identified by peptide mapping. *Mol. Pharmacol.* **47**, 956–964.

Villemagne V., Yuan J., Wong D. F., Dannals R. F., Hatzidimitriou G., Mathews W. B., Ravert H. T., Musachio J., McCann U. D. and Ricaurte G. A. (1998) Brain dopamine neurotoxcity in baboons treated with doses of methamphetamine comparable to those recreationally abused by humans: evidence from [^{11}C]WIN35428 positron emission tomgoraphy studies and direct in vitro determination. *J. Neurosci.* **18**, 419–427.

Vocci F., Chiang C. N., Cummings L. and Hawks R. (1995) Overview. medications development for the treatment of drug abuse. *NIDA. Res. Monogr.* **149**, 4–15.

Volkow N. D., Fowler J. S., Logan J., Wang G.-J., Hitzemann R., MacGregor R., Dewey S. L. and Wolf A. P. (1992) Decreased binding of 11-C-cocaine in the brain of cocaine addicts. *J. Nucl. Med.* **33**, 888.

Volkow N. D., Wang G. J., Fischman M. W., Foltin R. W., Fowler J. S., Abumrad N. N., Vitkun S., Logan J., Gatley S. J., Pappas N., Hitzemann R. and Shea C. E. (1997) Relationship between subjective effects of cocaine and dopamine transporter occupancy. *Nature* **386**, 827–830.

Wester P., Bateman D. E., Dodd P. R., Edwardson J. A., Hardy J. A., Kidd A. M., Perry R.H. and Singh G. B. (1985) Agonal status affects metabolic activity in nerve endings isolated from postmortem human brain. *Neurochem. Pathol* **3**, 169–180.

Wetli C. V. and Fishbain D. A. (1985) Cocaine-Induced psychosis and sudden death in recreational cocaine users. *J. Forensic Sci.* **30**, 873–880.

Wetli C. V., Mash D. C. and Karch S. B. (1996) Cocaine-associated agitated delirium and the neuroleptic malignant syndrome. *Am. J. Emerg. Med.* **14**, 425–428.

Wilson J. M., Levey A. I., Bergeron C., Kalasinsky K., Ang L., Peretti F., Adams V. I., Smialek J., Anderson W. R., Shannak K., Deck J., Niznik H. B. and Kish S. J. (1996) Striatal dopamine, dopamine transporter, and vesicular monoamine transporter in chronic cocaine users. *Ann. Neurol.* **40**, 428–439.