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Higher Binding of the Dopamine D_3 Receptor-Preferring Ligand [^{11}C]-(+)-Propyl-Hexahydro-Naphtho-Oxazin in Methamphetamine Polydrug Users: A Positron Emission Tomography Study

Isabelle Boileau,[1,2,4,5,7] **Doris Payer,**[1,2,7] **Sylvain Houle,**[2] **Arian Behzadi,**[1,2,7] **Pablo M. Rusjan,**[2] **Junchao Tong,**[1,2,3,7] **Diana Wilkins,**[11] **Peter Selby,**[4,7,9,10] **Tony P. George,**[5,7] **Martin Zack,**[6,8,9] **Yoshiaki Furukawa,**[3] **Tina McCluskey,**[1,2,3] **Alan A. Wilson,**[2] **and Stephen J. Kish**[1,2,3,7,8]

[1]Addiction Imaging Research Group, [2]Vivian M. Rakoff PET Imaging Centre, [3]Human Brain Laboratory, [4]Addictions, [5]Schizophrenia, and [6]Clinical Neuroscience Programs, Centre for Addiction and Mental Health, Toronto, Ontario, Canada M5T 1R8, Departments of [7]Psychiatry, [8]Pharmacology, [9]Public Health Sciences, and [10]Family and Community Medicine, University of Toronto, M5S 1A1 Toronto, Ontario, Canada, and [11]Center for Human Toxicology, University of Utah, Salt Lake City, Utah 84112

Positron emission tomography (PET) findings suggesting lower D_2-type dopamine receptors and dopamine concentration in brains of stimulant users have prompted speculation that increasing dopamine signaling might help in drug treatment. However, this strategy needs to consider the possibility, based on animal and postmortem human data, that dopaminergic activity at the related D_3 receptor might, in contrast, be elevated and thereby contribute to drug-taking behavior. We tested the hypothesis that D_3 receptor binding is above normal in methamphetamine (MA) polydrug users, using PET and the D_3-preferring ligand [^{11}C]-(+)-propyl-hexahydro-naphtho-oxazin ([^{11}C]-(+)-PHNO). Sixteen control subjects and 16 polydrug users reporting MA as their primary drug of abuse underwent PET scanning after [^{11}C]-(+)-PHNO. Compared with control subjects, drug users had higher [^{11}C]-(+)-PHNO binding in the D_3-rich midbrain substantia nigra (SN; +46%; $p < 0.02$) and in the globus pallidus (+9%; $p = 0.06$) and ventral pallidum (+11%; $p = 0.1$), whereas binding was slightly lower in the D_2-rich dorsal striatum (approximately −4%, NS; −12% in heavy users, $p = 0.01$) and related to drug-use severity. The [^{11}C]-(+)-PHNO binding ratio in D_3-rich SN versus D_2-rich dorsal striatum was 55% higher in MA users ($p = 0.004$), with heavy but not moderate users having ratios significantly different from controls. [^{11}C]-(+)-PHNO binding in SN was related to self-reported "drug wanting." We conclude that the dopamine D_3 receptor, unlike the D_2 receptor, might be upregulated in brains of MA polydrug users, although lower dopamine levels in MA users could have contributed to the finding. Pharmacological studies are needed to establish whether normalization of D_3 receptor function could reduce vulnerability to relapse in stimulant abuse.

Introduction

Animal data have long suggested that the neurotransmitter dopamine (DA) plays a role in several aspects of addiction, including addiction to the dopaminergic stimulant drugs methamphetamine (MA) and cocaine. However, despite years of intense investigation, no pharmacotherapy, DA related or other, has been approved for treatment of stimulant dependence (Kish, 2008). Whereas evidence points to low levels of DA and DA D_2 receptor number in stimulant users (Wilson et al., 1996; Volkow et al., 2001, 2009; Martinez et al., 2004, 2011; Lee et al., 2009; Wang et al., 2011), some animal and postmortem human data in cocaine users (Staley and Mash, 1996; Mash, 1997; Segal et al., 1997; Sokoloff et al., 2001) suggest that activity at the D_3 receptor,

Table 1. Subject demographic information

	Control subjects ($n = 16$)[a]	Methamphetamine users ($n = 16$)[a]	Group difference (p value)
Age (years)	28.43 ± 5.01 (16)	27.93 ± 5.66 (16)	0.32
Gender	14 (M)	12 (M)	0.33
Ethnicity	13 (W)	13 (W)	0.67
Weight (kg)	74.95 ± 17.96 (16)	80.53 ± 14.52 (16)	0.21
Years of education	16.68 ± 2.62 (16)	12.75 ± 2.56 (16)	*<0.01*
Premorbid IQ (NART)	117.43 ± 5.77 (12)	115.4 ± 4.50 (16)	0.31
Beck Depression Inventory	1.18 ± 2.16 (16)	6 ± 6.83	*0.02*
Inventory of Depressive Symptomatology	4.13 ± 4.45 (15)	10 ± 8.59 (16)	*0.02*
Nicotine smokers (>5 cigarettes d)	1 (16)	7 (16)	*0.02*
Cigarettes per day	0.7 ± 1.65 (16)	4.8 ± 4.8 (16)	*<0.01*
Cannabis (≥1 time per month)	3 (16)	9 (16)	*0.02*
Alcohol use (>14 drinks per week)	0 (16)	1 (16)	0.3
Purdue Pegboard Task			
Dominant	15.5 ± 1.89 (16)	13.93 ± 2.97 (16)	*0.03*
Nondominant	14.62 ± 1.70 (16)	14 ± 1.93 (16)	0.34
Both hands	12.31 ± 1.62 (16)	11.5 ± 1.5 (16)	0.15
Trails			
A	21.83 ± 4.96 (12)	25.46 ± 4.50 (15)	*0.03*
B	45.75 ± 9.70 (12)	51.06 ± 11.73 (15)	0.1
Digit symbol substitution	78.6 ± 12.2 (10)	59.2 ± 14.8 (15)	*<0.01*
Written	63.0 ± 11.4 (10)	54.8 ± 8.6 (15)	*0.02*
Eysenck Personality Inventory			
Extroversion	10.5 ± 5.05 (16)	14.07 ± 4.32 (14)	*<0.01*
Neuroticism	4.87 ± 3.24 (16)	9.57 ± 3.56 (14)	*<0.01*
Impulsivity	3.31 ± 1.66 (16)	5.07 ± 2.05 (14)	*0.02*

M, Male; W, White.

[a]Data are mean ± SD (n).

Italics indicate $p < 0.05$.

a member of the D$_2$-like receptor family, may be pathologically increased in addiction. If correct, a therapeutic strategy aimed at a generalized increase in DA signaling might successfully address a D$_2$ deficiency but exacerbate an already exaggerated D$_3$-related process.

Over the past 20 years, interest in the novel D$_3$ receptor, for which the function is still unknown, has developed in large part because of the strikingly high preferential expression of the receptor in limbic brain areas associated with reward and motivation (e.g., ventral limbic striatum) (Sokoloff et al., 1990; Murray et al., 1994), whereas the D$_2$ receptor is distributed uniformly throughout the striatum (Sokoloff et al., 1990). In animal models, D$_3$-selective antagonists decrease drug-seeking behavior, raising the possibility that this receptor modulates motivation to self-administer drugs (Le Foll et al., 2005; Heidbreder and Newman, 2010). Animal data also suggest that sensitization to DA-elevating drugs [long hypothesized to explain stimulant addiction in humans (Robinson and Berridge, 1993)] secondary to repeated dopaminergic stimulation coincides with increased D$_3$ receptor expression in ventral, but also dorsal striatal, regions that do not normally express high levels of D$_3$ (Bordet et al., 1997). Together, the findings tentatively suggest that increased transmission at the D$_3$ receptor in limbic striatal and ectopic regions could underlie some aspects of psychostimulant addiction.

With the recent development of a D$_3$-preferring positron emission tomography (PET) radiotracer, [^{11}C]-(+)-propyl-hexahydro-naphtho-oxazin ([^{11}C]-(+)-PHNO) (Wilson et al., 2005), it has become possible to investigate the contribution of the D$_3$ receptor in living human brain. [^{11}C]-(+)-PHNO binding (~20-fold selectivity for D$_3$ over D$_2$) can be interpreted in a region-dependent manner, with binding in dorsal striatum (high D$_2$/low D$_3$ expression) more likely reflecting D$_2$ receptor availability and binding in hypothalamus and substantia nigra (SN) reflecting predominantly D$_3$ availability. The ventral pallidum

(VP) and globus pallidus (GP) are areas of mixed D$_2$/D$_3$ binding where the D$_3$ fraction has been estimated to represent 75 and 65%, respectively (Tziortzi et al., 2011).

Based on the above data, we tested the hypothesis that MA users would have above-normal [^{11}C]-(+)-PHNO binding in a D$_3$-rich brain area (midbrain/SN) and decreased binding a high D$_2$/low D$_3$-expressing brain area (dorsal striatum) .

Materials and Methods

Subjects. Sixteen healthy and 16 MA-using volunteers participated in a PET imaging study approved by the Centre for Addiction and Mental Health Research Ethics Board. Brain PET measures of the vesicular monoamine transporter have been reported previously for some of these cases (Boileau et al., 2008).

All participants underwent a comprehensive medical and psychiatric screening interview and completed a comprehensive drug-history questionnaire (structured and open ended, locally developed), which included questions about drug-use frequency, typical dose and route of administration, years of use, recent drug use, withdrawal symptoms, time spent in drug-related activities (e.g.: using, seeking, recovering from drug effects), number of failed attempts to quit, impact on daily activities, and readiness to change use. MA users and control subjects were healthy males or females (age, 19–45 years) and were free of significant medical conditions and current or previous *Diagnostic and Statistical Manual of Mental Disorders*, fourth revision (DSM-IV) Axis I disorders (First et al., 1996) (excluding stimulant abuse/dependence in the MA group and nicotine dependence in both groups). Study inclusion criteria for the MA group included the following: (1) self-reported use of MA as the primary drug of abuse; (2) meeting DSM-IV criteria for MA abuse or dependence (all subjects also met proposed DSM-V criteria for "amphetamine use disorder"); (3) testing positive for MA in scalp hair; and (4) no current (12 months) self-reported abuse of or dependence on drugs other than MA (except nicotine). On screening day, subjects completed mood scales, a general IQ test, and the Eysenck Personality Inventory (Eysenck, 1953) (Table 1).

PET imaging session and region-of-interest analyses. MA users were asked to withhold all illicit drug use for a minimum of 14 d before the scan. On the day of the scan, all subjects were required to test negative on a urine drug screen (9-Drug Test Panel; BTNX) and complete the Profile of Mood States (POMS) questionnaire (McNair et al., 1992), visual analog scales (VAS) measuring drug craving, and the Purdue Pegboard Task of motor dexterity (Lafayette Instrument Company). A short battery of neuropsychological tests was administered after the PET scan (Table 1).

On a separate session (<7 d after the PET scan), all subjects received an oral dose of dextro-amphetamine (0.4 mg) and reported mood and drug-related feelings (results of this study will be reported separately).

[^{11}C]-(+)-PHNO synthesis and image acquisition protocols on the CPS-HRRT neuro-PET camera system (Siemens Medical Imaging) were described in detail previously (Graff-Guerrero et al., 2008). Scans were initiated after bolus injection of [^{11}C]-(+)-PHNO (mean dose, 303.4 MBq; specific activity, 1263.89 mCi/μmol; mean mass, 2.3 μg). Raw data were reconstructed by filtered-back projection. Spin echo proton-density weight magnetic resonance images (MRIs; slice thickness, 2 mm; repetition time, >5300 ms; echo time, 13 ms; flip angle, 90°; number of excitations, 2; acquisition matrix, 256 × 256; FOV, 22 cm) were obtained (Signa 1.5T MRI scanner; General Electric Medical Systems) for region-of-interest (ROI) delineation.

ROI delineation and time activity curve analyses were performed using in-house image analysis software for automated quantification of PET data [ROMI; details by Rusjan et al. (2006)]. Bilateral subcompartments of the striatum, including sensorimotor striatum (SMST), associative striatum (AST), and limbic striatum (LST), were automatically segmented (Rusjan et al., 2006) as described by Martinez et al. (2003). The (whole) GP was delineated with the procedure described and validated by Rusjan (2008). The ROI identified as the midbrain SN corresponded to contiguous midbrain gray matter voxels extending from planes $z = -4$ to $z = -14$ on six consecutive transverse slices in stereotaxic space (2 mm, MNI space). Identification of midbrain gray matter voxels within this region was performed by using the automated procedure described by Rusjan et al. (2006). The automatically selected VP covered approximately five coronal slices starting at the interhemispheric anterior commissural connection and was defined laterally and medially as described by Tziortzi et al. (2011). Cerebellar cortex (excluding vermis, lobules IX and X) served as the reference region. [^{11}C]-(+)-PHNO time activity curves were obtained from dynamic data, and specific binding (BP$_{ND}$) was estimated in each ROI using the simplified reference tissue method (SRTM) (Lammertsma and Hume, 1996). Parameter estimation was performed with PMOD (version 2.8.5; PMOD Technologies).

Voxel-wise parameter estimation. Voxel-wise parameter estimation of [^{11}C]-(+)-PHNO binding was generated using the basis function implementation of SRTM (Lammertsma and Hume, 1996) with the tissue time activity curve of cerebellar cortex as the reference region. Normalized BP$_{ND}$ maps (SPM2; Wellcome Trust Centre for Neuroimaging, London, UK) were statistically investigated to assess significant contrasts between groups at every voxel, using independent sample t test analysis (SPM8). The threshold for significant clusters was set to a family-wise error (FWE) corrected $p < 0.05$. This approach is aimed at detecting differences in neuroreceptor ligand binding at the voxel level, with no a priori anatomical hypothesis, and enables circumvention of some limitations of ROI placement, as well as investigation of regions not included in our ROI template (e.g., the hypothalamus).

Statistical approach. Group comparisons of [^{11}C]-(+)-PHNO binding across ROIs were conducted using standard repeated-measures ANOVAs or ANCOVAs (ROIs × group). When indicated, sphericity corrections were made with Greenhouse-Geisser adjustments. Least significant difference t tests, Bonferroni corrected for planned comparisons, were applied to determine the significance of regional differences in BP$_{ND}$ between groups. The ratio of SN [^{11}C]-(+)-PHNO BP$_{ND}$ [100% D$_3$ (Tziortzi et al., 2011)] to SMST [^{11}C]-(+)-PHNO BP$_{ND}$ [0% D$_3$ (Tziortzi et al., 2011)] was estimated as an index of individual D$_3$ levels, and t tests were used to assess group differences. One-tailed tests were selected to investigate potential decreased binding in D$_2$-rich dorsal striatum. Relationships between continuous variables were analyzed with the

Table 2. Co-used substances

	Control subjects (n = 16)	Methamphetamine users (n = 16)
Methamphetamine/amphetamine	0%	100%
	0 (11)	15 (15)
Cocaine/cocaine metabolites[a]	0%	80%
	0 (11)	12 (15)
MDMA/MDA/MDEA[a]	0%	53.3%
	0 (11)	8 (15)
Benzopdiazepine[a]	0%	33%
	0 (11)	5 (15)
Morphine/codeine[a]	0%	46%
	0 (11)	7 (15)
THC (>1 month)[b]	18.7%	56.2%
	3 (16)	9 (16)
Ketamine[b]	0%	25%
	0 (16)	4 (16)

[a]Hair.
[b]Self-report.
MDMA, 3,4-Methylenedioxymethamphetamine; MDA, 3,4-Methylenedioxyamphetamine; MDEA, 3,4-Methylenedioxy-*N*-ethylamphetamine.

Table 3. Methamphetamine use patterns

	Methamphetamine use[a]
Years of MA use	5.1 ± 2.7; 2–11; 4 (16)
Days since last use	18.5 ± 20.5; 6–90; 14 (16)
Typical frequency (days/week)	2.1 ± 1.1; 1–5; 2 (16)
*Binge in the last 30 d[b]	5.1 ± 2.7; 0–10; 5.5 (16)
Route	8 (16) intravenous, smoke; 8 (16) nasal, oral
Estimated dose (mg)	325 ± 167; 100–500; 300 (12)

[a]Data are mean ± SD; range; median (*n*).
[b]Period of 2–3 d of use.

Pearson product moment correlation coefficient and Spearman's rank test for categorical data.

Results

Demographic characteristics and drug profiles

MA polydrug users matched control subjects on age, gender, and ethnicity but had slightly lower education levels. They scored lower than control subjects on the Purdue test of motor dexterity and on tests of working memory and attention, but groups did not significantly differ with respect to estimated premorbid IQ (NART) (Table 1). MA polydrug users self-rated as being more impulsive, and although not clinically depressed, had significantly greater self-reported depressive symptoms. They also used more cannabis and tobacco but did not report drinking more alcohol (Table 1).

Hair analysis confirmed use of MA in all subjects with the exception of one MA user, who did not have scalp hair but provided an MA-positive urine sample at interview. Although MA-using subjects reported MA as the primary drug of abuse, hair analysis disclosed presence of other drugs in hair, particularly cocaine metabolites, confirming, as expected, that the MA users were polydrug users (Table 2).

The pattern of MA use was variable across the sample (Table 3). Sixty-three percent (10 of 16) of the sample was composed of "heavy" MA users who preferred smoking or injecting MA, often consumed doses >300 mg per occasion, and used MA >3 d/week for at least 3 months. The remaining 38%, "moderate" MA users, were lower-dose (<300 mg), bimonthly intranasal users. Ninety-three percent (15 of 16) had used MA at least once in the 30 d before the study, and only one case had used MA for <3 years at the time of the study. Cocaine use was prevalent in the sample based on hair data. Urine toxicology at the time of the screening (>14 d before the

scan) indicated that 5 of 12 MA users who tested positive for cocaine in hair recently used cocaine. Self-report indicated that the average use of cocaine in the last 30 d corresponded to 1.75 d.

PET $[^{11}C]$-(+)-PHNO BP$_{ND}$

An ANOVA investigating regional differences in $[^{11}C]$-(+)-PHNO binding across groups (with age as a covariate, as age related changes in D_2 (Rinne et al., 1993) and D_3 (Graff-Guerrero et al., 2009) binding have been suggested) yielded a significant Group × ROI interaction ($F_{(5,135)} = 3.35$; $p = 0.02$). Pairwise contrasts revealed that MA use was associated with significantly higher $[^{11}C]$-(+)-PHNO BP$_{ND}$ in D_3-rich SN (46%; corrected $p = 0.02$), with trends in mixed D_3/D_2 regions including the GP (11%; corrected $p = 0.06$) and VP (9%; corrected $p = 0.1$). Accounting for use of nicotine and cannabis did not change this finding (ANOVA with cannabis as covariate: $F_{(5,135)} = 1.98$, $p = 0.04$; ANOVA with nicotine as covariate: $F_{(5,130)} = 1.78$, $p = 0.05$). In subcompartments of the D_2-rich dorsal striatum, only a small nonsignificant difference in BP$_{ND}$ was observed (~4%) (Fig. 1). However, a separate ANOVA entering severity of MA use as a grouping variable and age as a covariate revealed that heavy but not moderate MA use was associated with significantly lower $[^{11}C]$-(+)-PHNO binding ($F_{(10,130)} = 3.89$; $p = 0.001$). Relative to controls, decreased $[^{11}C]$-(+)-PHNO binding was maximal in the SMST (-11.4%; $p = 0.04$, uncorrected for one-tailed comparison) but also occurred in the whole dorsal striatum [one-way ANOVA; $F_{(2,30)} = 2.87$; $p = 0.04$ (-11%; $p = 0.03$)]. This effect was not significant in the LST ($p > 0.05$). The pairwise comparison also revealed that the increase in D_3 receptor binding in the midbrain SN was driven by severity of use since heavy but not moderate use was associated with above control $[^{11}C]$-(+)-PHNO BP$_{ND}$ ($F_{(10,130)} = 3.89$; $p = 0.001$; $p = 0.04$). Investigating differences in D_3/D_2 ratios (SN/SMST $[^{11}C]$-(+)-PHNO BP$_{ND}$) between groups, MA users had a 55% greater D_3 to D_2 binding fraction when compared with controls ($p = 0.004$), with heavy (72%; $p = 0.001$) but not moderate (26%; $p = 0.2$) users having ratios significantly different from controls. Standard uptake values for cerebellum did not differ between MA and controls ($p > 0.05$). $[^{11}C]$-(+)-PHNO mass injected was not significantly different between groups ($p > 0.3$), and no significant correlation between mass and $[^{11}C]$-(+)-PHNO BP$_{ND}$ was observed in any region investigated ($p > 0.1$).

To investigate the functional significance of differences in $[^{11}C]$-(+)-PHNO binding, we tested for relationships between regional BP$_{ND}$ and self-report measures taken at baseline and after a priming dose of amphetamine. In the MA group, lower regional $[^{11}C]$-(+)-PHNO binding in dorsal striatum was associated with greater severity of MA use (years of use: AST, $r = -0.6$, $p = 0.01$; SMST, $r = -0.5$, $p = 0.01$; severity rank: AST, $\rho = -0.6$, $p = 0.01$; SMST, $\rho = -0.8$, $p < 0.001$) and daily nicotine use (SMST, $r = -0.5$, $p = 0.01$). On the other hand, greater $[^{11}C]$-(+)-PHNO binding in dorsal striatum was associated with higher (negative) subjective responses to the low-dose amphetamine challenge (VAS "mind racing": AST, $r = 0.6$, $p = 0.01$; SMST, $r = 0.6$, $p = 0.01$). This effect was also observed in the
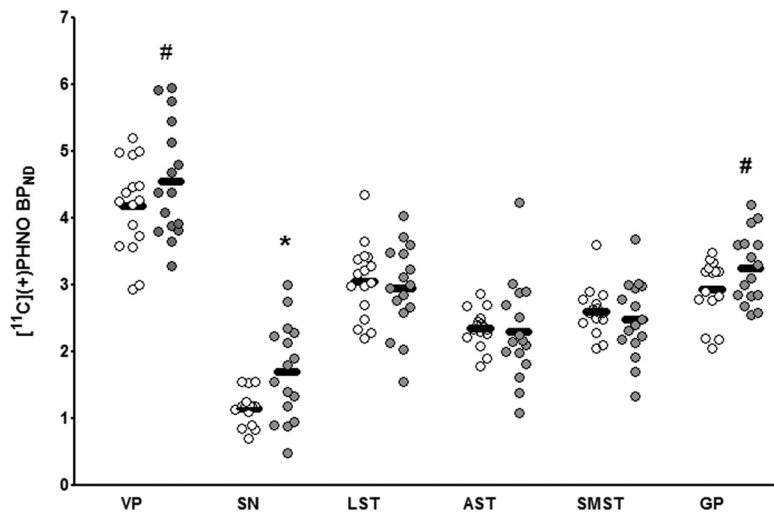
Figure 1. Regional $[^{11}C]$-(+)-PHNO BP$_{ND}$ in MA users ($n = 16$; gray circles) and in control subjects ($n = 16$; white circles). *$p < 0.05$, corrected; #$p < 0.1$, corrected.

overall sample after covarying out the effect of Group (partial correlation with group as a covariate) such that, in the dorsal striatum, higher binding was associated with greater drug-related (negative) feelings (VAS mind racing: AST, $r = 0.6$, $p = 0.005$; SMST, $r = 0.7$, $p = 0.001$). Higher $[^{11}C]$-(+)-PHNO BP$_{ND}$ in the D_3-rich SN predicted motivation to use methamphetamine and, to a lesser extent, to amphetamine-induced "rush" in MA users (VAS "drug wanting": $r = 0.8$, $p = 0.001$; VAS "rush": $r = 0.4$, $p = 0.06$) and in the sample overall (VAS drug wanting: $r = 0.6$, $p = 0.001$; VAS rush: $r = 0.5$, $p = 0.01$).

In the voxel-wise statistical analyses (SPM8), we identified small clusters of greater $[^{11}C]$-(+)-PHNO BP$_{ND}$ in MA users relative to controls in an area corresponding to the midbrain (SN/VTA) (Fig. 2A). Small, noncontiguous clusters of increased binding were also detected in the basal forebrain (around VP); however, those were below the significance threshold (data not shown). No clusters of significantly lower $[^{11}C]$-(+)-PHNO BP$_{ND}$ survived correction for multiple comparisons; however, using a more liberal threshold, differences between groups were identified at discrete sites within the dorsal striatum, with the most significant cluster of lower $[^{11}C]$-(+)-PHNO BP$_{ND}$ in the MA group occurring in the postcommissural putamen (SMST) and the caudate (AST) (Fig. 2B).

Discussion

To our knowledge, this is the first *in vivo* brain imaging study of the D_3 receptor in drug-abusing humans. We found evidence for greater DA D_3 receptor binding in brain of MA users. As the D_3 receptor has been implicated in drug-taking behavior, this finding is relevant to proposed therapeutic strategies targeting D_3-selective antagonism.

Our findings are discussed below in the context of assessing $[^{11}C]$-(+)-PHNO binding in a region-dependent manner, with binding in dorsal striatum more likely reflecting D_2 receptor availability and binding in SN reflecting predominantly D_3 availability (Tziortzi et al., 2011).

$[^{11}C]$-(+)-PHNO binding in D_2-rich striatum is slightly decreased

PET studies have shown that addictive disorders in humans are associated with low striatal $D_{2/3}$ receptor binding (Volkow et al., 2001; Martinez et al., 2004; Lee et al., 2009). In our sample, which included heavy and moderate MA users, we detected only mini-
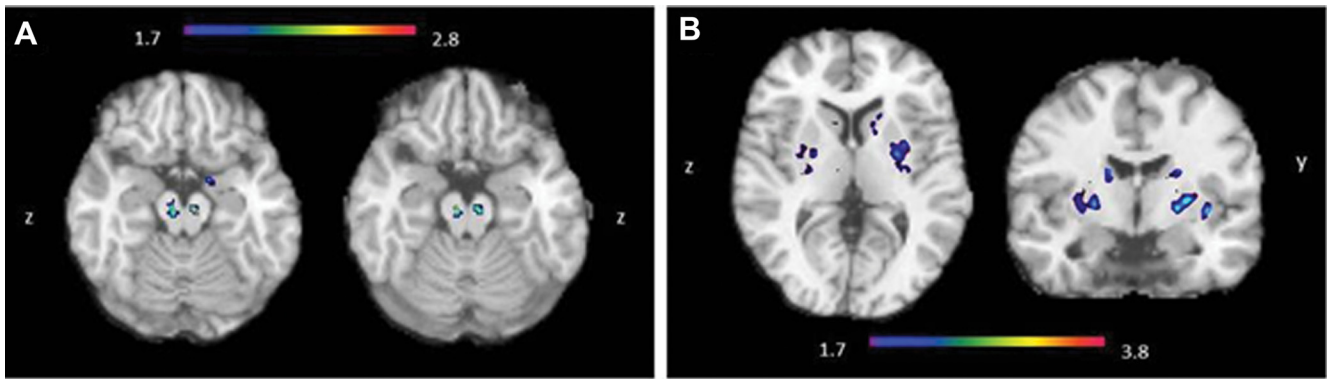
Figure 2. **A**, Voxel-wise group comparison illustrating higher [^{11}C]-(+)-PHNO BP$_{ND}$ in the midbrain of MA-abusing than control subjects ($t_{max} = 2.79$; $p = 0.01$; MNI coordinates: 8, −10, −12) and cluster above the $p < 0.05$ significance threshold when using the random field FWE correction for multiple comparisons within a small volume (10-mm-radius sphere; MNI coordinates: 10, −18, −12). **B**, Voxel-wise group comparison illustrating lower [^{11}C]-(+)-PHNO BP$_{ND}$ in the striatum of MA-abusing than control subjects ($t_{max} = 3.90$; $p = 0.04$, uncorrected; $k_E = 21$; MNI coordinates: 24, 2, 0). The image is thresholded at a probability uncorrected for multiple comparisons.

mal nonsignificant decreases in D$_{2/3}$ receptor binding in D$_2$-rich dorsal striatum; however, in line with previous reports (Lee et al., 2009), MA abuse severity and chronicity were predictive of binding, such that heavy, but not moderate, use of MA was associated with significantly lower binding in dorsal striatum. The regional extent of the finding as indicated by both the ROI and voxel-wise approach, was comparable to that in previous PET studies in MA users (Lee et al., 2009), showing maximal effect in dorsal striatum (including SMST and AST) versus LST.

Overall, the differences in D$_{2/3}$ receptor binding in dorsal striatum were slightly below values reported in the literature. Although lower cumulative exposure to MA in our sample can reasonably explain our marginal finding, intrinsic properties of [^{11}C]-(+)-PHNO could also partly account for the smaller magnitude of effect. As animal studies show that repeated exposure to DA-elevating drugs increases D$_3$ receptor population in areas previously almost entirely devoid of D$_3$ receptors [i.e., the dorsal striatum (Bordet et al., 1997)], an ectopic upregulation of the D$_3$ receptor in dorsal striatum could have masked potential loss of D$_2$ receptor binding. Alternatively, differences in DA levels (K_d) across groups might have confounded measurement of receptor density (B_{max}). As [^{11}C]-(+)-PHNO is a high-affinity agonist ligand, it is more sensitive (vs [^{11}C]raclopride) to modulation by DA (Willeit et al., 2008; Shotbolt et al., 2012). It is therefore possible that low levels of DA in brain of MA users (Wilson et al., 1996; Moszczynska et al., 2004) could lead to greater available receptor sites for binding, hence increasing BP$_{ND}$ and masking the presumed loss.

The functional significance of low D$_2$ receptor binding in addiction is unclear, and, concurring with other studies (Martinez et al., 2004), we did not find that decreased D$_2$ receptor density was related to drug-craving or positive effects of amphetamine. However, partly in line with the notion that greater D$_2$ DA stimulation is associated with negative effects of stimulants (Volkow et al., 1999), which could protect against further drug use, higher striatal D$_2$ receptor binding was related to "racing thoughts," a negative effect of amphetamine associated with anxiety (and hypomanic state). Thus, in MA users, lower aversive side effects of amphetamine, presumably mediated by decreased D$_2$ stimulation, could contribute to MA abuse.

[^{11}C]-(+)-PHNO binding in D$_3$-rich compartments is increased

In contrast to the D$_2$ findings, our data suggest that brain D$_3$ receptor density in the D$_3$-rich SN, but also in the mixed D$_2$/D$_3$

GP/VP, might be higher in psychostimulant users. Although lower DA levels (Moszczynska et al., 2004) might, in principle (see Shotbolt et al., 2012), explain increased [^{11}C]-(+)-PHNO binding in the MA users, this possibility is less likely in view of the results of some (though not all; see Richtand et al., 2001) preclinical studies showing increased D$_3$ receptor levels and mRNA after stimulant exposure in nucleus accumbens (where D$_3$ predominates) and extrastriatal areas (SN, VP, and GP) (Morissette et al., 1998; Quik et al., 2000), as well as postmortem brain investigations reporting that D$_3$ receptor binding is higher in cocaine overdose fatalities (Staley and Mash, 1996; Mash, 1997; Segal et al., 1997; Sokoloff et al., 2001). Increased [^{11}C]-(+)-PHNO binding may be a consequence of D$_3$ receptor upregulation in GABAergic neurons containing substance P and dynorphin (Frankel et al., 2008), as concentrations of dynorphin in brain and plasma brain-derived neurotrophic factor, the latter considered to regulate D$_3$ expression (Guillin et al., 2001), are elevated in human stimulant users (Kim et al., 2005; Frankel et al., 2007, 2008).

Study limitations

Currently, use of [^{11}C]-(+)-PHNO is the only method available to quantify D$_3$ receptors *in vivo*. In this regard, study limitations include use of a radioligand lacking absolute specificity for the DA D$_3$ receptor and problematic interpretation of binding data in areas that contain both D$_2$ and D$_3$ receptors. Recent studies, however, have suggested that the [^{11}C]-(+)-PHNO signal can be regionally divided into a "relatively pure" D$_3$ component (the SN and hypothalamus, in which 100% of [^{11}C]-(+)-PHNO binding is to D$_3$) and a D$_2$ component [the dorsal striatum, where 100% of the binding is to the D$_2$ (Tziortzi et al., 2011)]. This characterization of the signal makes it possible to draw some conclusions, albeit highly region dependent, from our findings.

Our failure to find group differences in D$_{2/3}$ receptor binding in the LST, a region where the relative fraction of D$_3$ to D$_2$ receptor (26%) is larger than that in the dorsal striatum (<6%), could, for example, be attributed to the above-mentioned limitation. Specifically, the possibility that coexisting D$_2$ and D$_3$ receptor systems have opposing functional responses to DA-elevating drugs (Levesque et al., 1995) could have canceled out an effect in either direction. An alternative explanation is the fact that the smaller LST is more prone to partial volume effects and higher variability of binding values, which together increase noise and limit measurements in this area.

A practical issue also to be considered is the fact that at the doses of [^{11}C]-(+)-PHNO used in the current study and in stud-

ies of other groups (Searle et al., 2010; Tziortzi et al., 2011), injected mass may lead to receptor occupancies higher than tracer doses, which could result in an underestimation of BP_{ND} in both groups and possibly decrease ability to detect a difference. Importantly, however, there were no significant differences in mass injected between groups or correlations between BP_{ND} and mass injected, making it unlikely that the finding of a group difference is attributable to a mass effect (see Shotbolt et al., 2012, their supplementary information). A methodological caveat of using [^{11}C]-(+)-PHNO that needs to be mentioned is the possibility that the result is biased by differential specific binding in the cerebellum. This potential bias is unlikely to explain our finding since cerebellar standard uptake values were not significantly different between groups; furthermore, the region selected as cerebellar input function excludes areas reported to contain D_3 receptors (vermis, lobules IX and X) (Murray et al., 1994). Another issue for consideration is the potential generic confound of other drugs used on D_3 receptor binding. Although subjects reported MA as the primary drug of abuse, drug hair analysis and self-report data showed, not unexpectedly, that drugs other than MA (nicotine, cocaine, and opiates) were used (and sometimes not reported) by subjects, which might well have influenced DA receptor expression. However, in light of findings that low D_2 receptor binding is a feature of different classes of abused drugs (Volkow et al., 2009), it could be argued that high brain D_3 might also be a characteristic across different drugs of abuse, a possibility that could be addressed in future investigations. Finally, we acknowledge that although animal studies suggest that heightened D_3 expression in MA users could reasonably be caused by sustained MA-induced dopaminergic stimulation, this difference could have predated drug use.

Possible functional implication of increased D₃ and conclusion

Notwithstanding the above limitations, our brain imaging findings do suggest that the DA D_3 receptor might be upregulated in polydrug MA users. The brain area involved includes at least the D_3-rich SN but might also involve GP/VP brain regions and striatum in which a D_3 increase might have been masked by a D_2 reduction. Although D_3 receptors are both reciprocal autoreceptors and heteroreceptors (Sokoloff et al., 1990), evidence of D_3 receptor mRNA induction and increased D_3 receptor binding in animals pretreated with DA-elevating drugs has suggested that the newly synthesized receptors are likely to occur in medium-sized spiny neurons containing D_1 receptors, dynorphin, and substance P (vs D_2 receptors and enkephalin), since their appearance coincided with increased prodynorphin mRNA (Bordet et al., 1997, 2000). However, this finding does not exclude the possibility of some increase in D_3 receptor occurring on SN DA cells for which there is, as yet, no known physiological role (Davila et al., 2003). The clinical implications of the increase (presumably on striatonigral projections) depend on the actual function of the D_3 receptor, still to be determined in mammalian brain, but might be related to a hypersensitive DAergic response to DA stimulation. Thus, studies investigating the effects of D_3 receptor induction find that increased D_3 receptor mRNA parallels the appearance of locomotor sensitization to a DA-elevating challenge (an animal model of addiction), possibly through increased inhibition of GABAergic neurons via stimulation of D_3 receptors in SN pars reticulata (Bordet et al., 1997, 2000; Guillin et al., 2001; Le Foll et al., 2003). Overall, increased D_3 receptor function in areas of the SN, VP, and GP, which receive afferent ventral striatum projections (Haber et al., 2000), could modify the functional

system responsible for the output of the limbic striatum and therefore modulate motivation to use drugs (Sokoloff et al., 2001). Indeed, across MA users, we found a robust relationship between [^{11}C]-(+)-PHNO binding in midbrain SN and self-reported drug wanting after a priming dose of amphetamine, suggesting that D_3 receptor activation could contribute to craving (and relapse). The finding is consistent with attenuation of drug seeking, self-administration, and cue- and stress-induced reinstatement after highly selective D_3 receptor antagonists (for review, see Heidbreder et al., 2005), together suggesting that a D_3 receptor increase might contribute to the addicted state in humans.

To summarize, our brain imaging data suggest that the D_3 receptor, unlike the D_2 receptor, might be upregulated in brain of MA users. Preclinical findings suggest that D_3 upregulation might contribute to the addicted state, but pharmacological studies in the human using D_3-specific antagonists and agonists are needed to establish the clinical significance of our observations.

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