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MDMA (ecstasy) use is associated with reduced BOLD signal change during semantic recognition in abstinent human polydrug users: a preliminary fMRI study

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Abstract

3,4-methylenedioxymethamphetamine (MDMA) users have impaired verbal memory, and voxel-based morphometry has shown decreased grey matter in Brodmann area (BA) 18, 21 and 45. Because these regions play a role in verbal memory, we hypothesized that MDMA users would show altered brain activation in these areas during performance of a functional magnetic resonance imaging (fMRI) task that probed semantic verbal memory. Polysubstance users enriched for MDMA exposure participated in a semantic memory encoding and recognition fMRI task that activated left BA 9, 18, 21/22 and 45. Primary outcomes were percent blood oxygen level-dependent signal change in left BA 9, 18, 21/22 and 45, accuracy and response time. During semantic recognition, lifetime MDMA use was associated with decreased activation in left BA 9, 18 and 21/22 but not 45. This was partly influenced by contributions from cannabis and cocaine use. MDMA exposure was not associated with accuracy or response time during the semantic recognition task. During semantic recognition, MDMA exposure was associated with reduced regional brain activation in regions mediating verbal memory. These findings partially overlap with previous structural evidence for reduced grey matter in MDMA users and may, in part, explain the consistent verbal memory impairments observed in other studies of MDMA users.

Key words

drug abuse; ecstasy (MDMA); fMRI; neuroimaging; semantic memory; verbal memory

Introduction

After a period of decline, use of the recreational 'club drug' 3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy') has been rising in the United States since 2005, and recent data suggest that over 12 million Americans have used MDMA (Johnston, *et al.*, 2008; Substance Abuse and Mental Health Services Administration, 2003). Ecstasy remains highly popular throughout the world, especially in North America, Western Europe and Oceania (United Nations Office on Drugs and Crimes, 2008).

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Numerous animal studies support MDMA-induced long-term serotonergic (5-HT) alterations through degeneration of presynaptic axon terminals and depletion of brain 5-HT upon exposure to MDMA of adequate magnitude and chronicity (Gibb, *et al*., 1990; Green, *et al*., 2003; Ricaurte, *et al*., 2000). This has led to significant concerns regarding the neurotoxic potential of MDMA. However, loss of brain 5-HT markers can occur in the absence of axonal loss (Fantegrossi, *et al*., 2004; Wang, *et al*., 2004; Wang, *et al*., 2005), suggesting that MDMA may produce functional consequences for 5-HT neurotransmission even in the absence of axon degeneration. Animal data have also shown an association between MDMA use and long-term behaviour change, which may be mediated by chronic alterations in the 5-HT system (Easton and Marsden, 2006). These data raise significant concern about the possibility of chronic neurological effects of MDMA use in humans.

There is considerable evidence that MDMA induces alterations in the 5-HT system in humans, including reduced binding to the serotonin reuptake transporter (5-HTT) (de Win, *et al*., 2004; McCann, *et al*., 1998; McCann, *et al*., 2005; McCann, *et al*., 2008; Reneman, *et al*., 2001a; Semple, *et al*., 1999), reduced levels of the 5-HT breakdown product 5-hydroxyindoleacetic acid (5-HIAA) (McCann, *et al*., 2000) and upregulation of the 5-HT2A receptor (Reneman, *et al*., 2000b; Reneman, *et al*., 2000a; Reneman, *et al*., 2002). Evidence from human studies suggests that altered serotonergic neurotransmission is long-lasting (Curran and Verheyden, 2003; Reneman, *et al*., 2002), although some studies suggest that partial improvement may occur with long-term abstinence (Semple, *et al*., 1999; Thomasius, *et al*., 2003). Because the chronicity of the alterations induced by MDMA remains unclear, and the partial improvements observed with abstinence could reflect pre-existing differences, the issue of potential neurotoxicity in humans remains controversial.

Many, but not all (Back-Madruga, *et al*., 2003; Gouzoulis-Mayfrank, *et al*., 2005), studies have found evidence of impairment across multiple neurocognitive domains including verbal working memory (Jacobsen, *et al*., 2004), episodic memory (Morgan, 2000) and visual memory (Back-Madruga, *et al*., 2003) in subjects with a history of MDMA exposure. Recent meta-analyses found effect sizes ranging from small to substantial for impaired verbal memory and for short- and long-term memory impairment in MDMA users compared with non-MDMA using control subjects (Kalechstein, *et al*., 2007; Laws and Kokkalis, 2007). A recent prospective cohort study compared subjects who started using MDMA with non-MDMA polysubstance users, and found significantly reduced immediate and delayed verbal recognition on the Ray Auditory-Verbal Learning Test in the MDMA users (Schilt, *et al*., 2007).

Several associative studies have shown a relationship between neurocognitive deficits in MDMA users and underlying neural alterations. Bolla, *et al*. (1998) studied subjects abstinent from MDMA for a median of 4 weeks and found that verbal and visual memory deficits correlated with a reduction in levels of the 5-HT metabolite 5-HIAA. Using magnetic resonance spectroscopy, Reneman, *et al*. (2001d) found significant deficits in delayed word recall in MDMA users compared with control subjects that strongly associated with a reduced N-acetylaspartate/creatine ratio in the prefrontal cortex. McCann, *et al*. (1998) found that the relationship between 5-HTT binding and cognition seen in non-MDMA users was disrupted in MDMA users, suggesting that MDMA disrupts the relationship between 5-HT function and cognition. However, a causal link between neural alterations and neurocognitive dysfunction remains to be established.

Imaging studies have also supported the association between MDMA exposure and functional brain alterations. Reneman, *et al*. (2001c) found increased regional cortical blood flow and increased apparent diffusion coefficient of water in the globus pallidi of MDMA users who had been abstinent for at least 3 weeks. This is consistent with subchronic vasodilation in recently abstinent MDMA users. Using fluorodeoxyglucose positron emission tomography (FDGPET), Obrocki, *et al*. (1999) found significantly reduced left hippocampal metabolism in MDMA users compared with oncology controls. Two subsequent reports using similar techniques found reduced resting metabolism in bilateral caudate/putamen and in the left amygdala in MDMA users compared with oncology controls (Buchert, *et al*., 2001; Obrocki, *et al*., 2002). Using a N-back functional magnetic resonance imaging (fMRI) paradigm activating working memory, Daumann, *et al*. (2003) found significantly greater activation in the right superior parietal lobe and significantly reduced activation in left posterior cingulate cortex, bilateral inferior temporal gyri and bilateral angular gyri in recently abstinent relatively pure MDMA users compared with controls. Using a prospective technique in a similar study, Daumann, *et al*. found increased activation in parietal cortex compared with baseline assessment activation in subjects who continued to use MDMA or amphetamines. The activation was positively correlated with lifetime MDMA use (Daumann, *et al*., 2004). These studies support reduced resting brain metabolism in selected brain regions and alterations in regional brain activation that are associated with MDMA exposure.

Cowan, *et al*. (2003) used voxel-based morphometry (VBM) to study neocortical grey matter volume in MDMA polysubstance users and non-MDMA polysubstance users and found decreased grey matter concentrations in bilateral Brodmann area (BA) 18, left BA 21 and left BA 45. Interestingly, a PET study (Lee, *et al*., 2002) showed that similar regions (BA 9, 18, 21/22 and 45) were involved in semantic memory. Based on the findings of grey matter loss in the Cowan, *et al*. report, we hypothesized that BA 18, 21/22 (we included BA 22 because our fMRI task confluently activated both BA 21/22) and 45 would show altered activation during performance of a verbal memory task. We adapted the semantic memory task described by Lee, *et al*. (2002) with two goals in mind: 1) to produce activation in BA 9, 18, 21/22 and 45 to specifically probe the function of these brain regions and 2) to probe aspects of verbal memory that are potentially impaired in MDMA users. Our paradigm consisted of an encoding phase followed by a recognition phase after a very brief delay; as such, this paradigm tapped elements of semantic and working memory.

Our primary hypothesis was that increased lifetime episodes of MDMA use would be associated with altered percent blood oxygen level-dependent (BOLD) signal change in BA 18, 21/22 and 45 but not BA 9 during semantic recognition. Our secondary hypothesis was that increased lifetime episodes of MDMA use would be associated with poorer performance response times and increased errors on the semantic recognition task.

Methods

To enhance replicability and cross-study comparisons, data are presented (where applicable) as outlined by Poldrack (2008) in his guidelines for presenting a fMRI study.

Human subjects

We recruited 18 subjects for this study as part of a larger study of neuroimaging in MDMA use via advertisements requesting volunteers aged 18–35 with a history of MDMA or other drug use for an MRI study. In all, 2 of the 18 subjects were excluded for excessive motion and technical inadequacy of the volumetric scan. Consequently 16 (6 women and 10 men) right-handed polysubstance users (12 with a history of MDMA use) age 23.6 ± 2.7 years old completed the study, and all data reported are from those subjects. We enrolled polydrug users (versus enrolling specific MDMA/non-MDMA users) to conduct within-group exposure/outcome assays of drug exposure. We used Dutch words for the pseudo-word condition (see below) because Dutch words are easily pronounceable to English speakers but do not have semantic meaning. Therefore, exclusion criteria included an understanding of Dutch, lifetime use of between one and five tablets of MDMA (to exclude subjects with very low-level MDMA use), history of current or past substance or alcohol dependence, history of current or past Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition axis 1 psychiatric disorder (except substance-induced mood disorder or substance abuse), taking non-illicit psychoactive medications (whether prescription or over-the-counter) within 6 weeks before the study, endocrine abnormalities, history of loss of consciousness for over 30 min, contraindications to MR scanning, and a positive urine drug screen on the scan day. Because we were interested in chronic effects of MDMA exposure, all subjects were abstinent from MDMA for at least 2 weeks before the fMRI study day.

Ethics approval

The study protocol was approved by the Vanderbilt University Institutional Review Board and conformed to the World Medical Association's Declaration of Helsinki.

Experimental design

Task specification This study was a human within-subjects cross-sectional design assessing the association between self-reports of previous polydrug exposure and outcome variables for fMRI and behavioural response. To probe regional brain activation and behavioural response during semantic encoding, we modified a semantic encoding and recognition task developed for PET (Lee, *et al*., 2002). Because our primary goal was to measure regional brain activation, we used a block stimulus design. The fMRI task consisted of a word and pseudo-word encoding (learning) period and a word and pseudo-word recognition period. We used a block study design consisting of 12-s rest (fixation) periods (Figure 1) interspersed with 10-s blocks of word/pseudo-word encoding or word/pseudo-word recognition. During each encoding block, subjects were instructed to memorize a group of five English words or a group of five pronounceable pseudo-words in a 10-s encoding period. After a subsequent 12-s fixation period, subjects were presented with homophone pairs (e.g., pray vs prey) in which one of the homophones was novel (not presented during the preceding encoding block) during a 10-s recognition block and asked to correctly identify the word/pseudo-word they had learned during the encoding period. (Dutch words were used as pronounceable pseudo-words, and homophones corresponding to Dutch words were synthesized based on English pronunciation.) As such, the overall task involved visual and verbal memory encoding and recognition, working memory, and an element of decision-making and motor activation during the forced choice recognition task. Because all visual and motor components of encoding and recognition of words versus pseudo-words were identical except for the fact that the English words have meaning to the subjects, a contrast of regional brain activation during word encoding or recognition versus pseudo-word encoding or recognition was expected to isolate semantic memory.

Planned comparisons Planned comparisons were the association of percent BOLD signal change for semantic memory in each brain region (left BA 9, 18, 21/22 and 45) with previous drug use. Additional comparisons examined the association of drug use with recognition task variables of reaction time and accuracy.

Region of interest (ROI) analysis outcome variables

The primary outcome variables were percent BOLD signal change in the previously chosen brain regions of left BA 9, 18, 21/22 and 45. BA 18, 21/22 and 45 were chosen because of their role in semantic processing, and because the earlier VBM study (Cowan, *et al*., 2003) found reduced brain grey matter concentration in these regions (because VBM is a threshold based-assay), it is possible that BA 22 was also affected in our original VBM study. Because our present study evoked a confluent region of activation overlapping BA 21 and 22, we combined

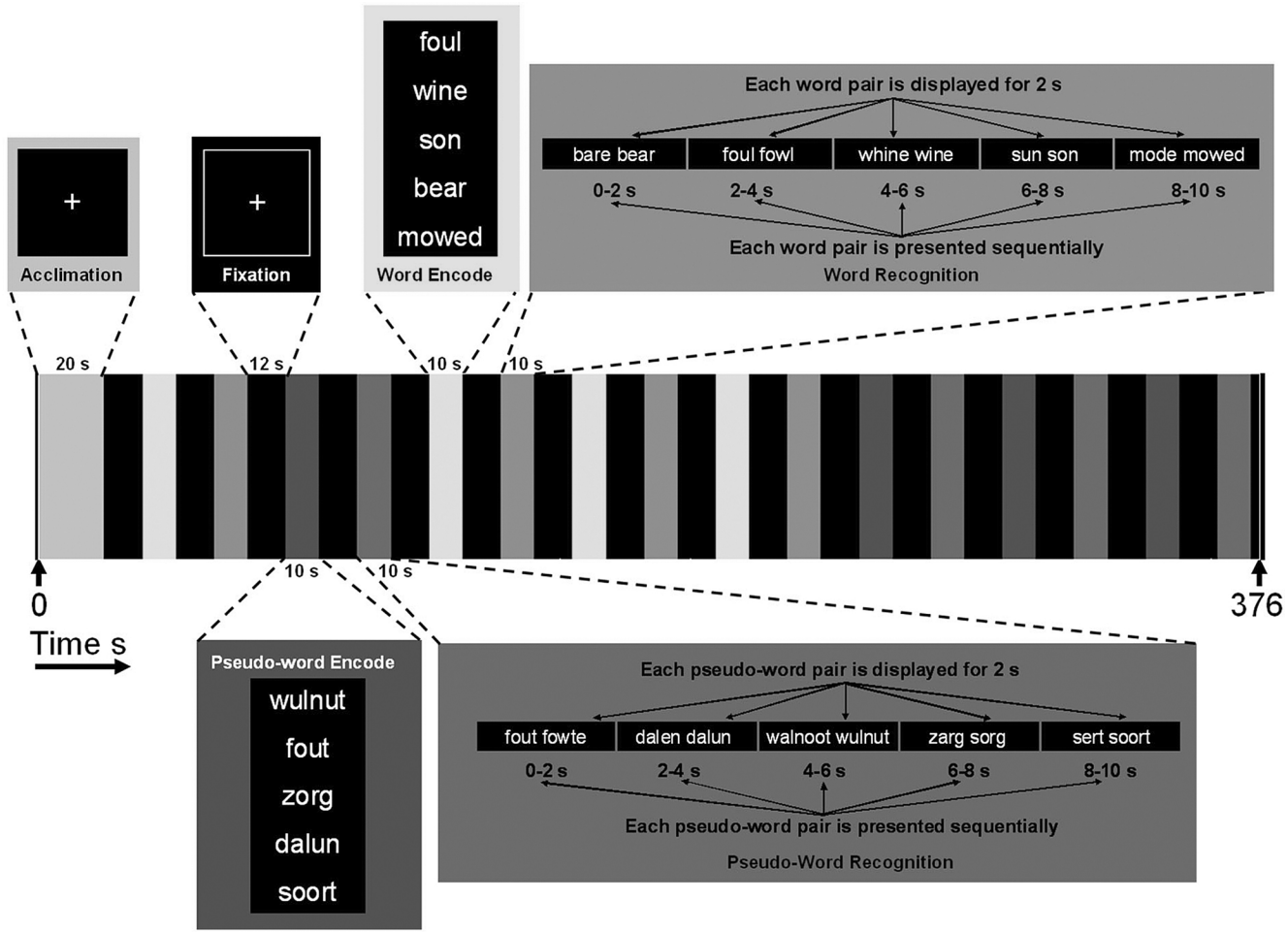

Figure 1 Semantic task. Subjects encoded a block of five words or pseudo-words followed by a recognition task using novel homophones as distracter images. Subjects were instructed to press a button corresponding to right or left screen location of previously encoded items. Semantic encode: activation during semantic encoding was defined as word encode – pseudo-word encode. Semantic recognition: activation during semantic recognition was defined as word recognition – pseudo-word recognition.

these regions in the analysis. BA 9 was included as a 'control' region because of its role in semantic processing and because the earlier VBM study did not find an association between MDMA use and BA 9 brain grey matter concentration. Percent BOLD signal change for each BA was calculated from the largest activated cluster (contiguous activated voxel group) in the BA. Percent BOLD signal change outcome for the largest cluster in each region was measured as semantic encode and semantic recognition. Percent BOLD signal change for semantic encode for a specific BA was calculated as a contrast of (percent BOLD signal change for word encode) − (percent BOLD signal change for pseudo-word encode). Similarly, percent BOLD signal change for semantic recognition was calculated as a contrast of (percent BOLD signal change for word recognition) − (percent BOLD signal change for pseudo-word recognition). These contrasts were devised based on the fact that word encode/recognition and pseudo-word encode/recognition differed only according to the semantic aspects of the stimuli. As shown in Figure 1, scanner runs were 376 s consisting of a 20-s fixation (white cross on black screen) followed by an initial encode session (five words or pseudo-words) displayed for 10 s, a fixation cross displayed for 12 s and a recognition phase (10 s). Each trial consisted of four encoding and four recognition blocks for words, and four encoding and four recognition blocks for pseudo-words. Subjects completed two trials (runs) of the task. The trials differed only by the random order of presentation of the encode and recognition events.

Behavioural performance

Secondary outcome variables were response time for correct responses for word recognition or pseudo-word recognition. Responses were analysed with regard to percentage correct

responses, reaction time or accuracy (number of omission and commission errors) for both the word and pseudo-word portions of the task. Because the behavioural measures do not permit a simple subtraction of effects as analysed for the regional BOLD signal change data, we reported word recognition and pseudo-word recognition measures separately and not as a semantic construct. As such, behavioural measures were analysed in relation to BOLD signal change during word or pseudo-word recognition but not for the derived semantic measure. Of the 16 subjects completing imaging, behavioural data for the recognition phase were not obtained for one subject. Although previous reports have found no association between MDMA use and verbal intelligence (Jager, *et al*., 2007; Jager, *et al*., 2008; Schilt, *et al*., 2007), we assessed verbal intelligence quotient (IQ) using Wechsler Abbreviated Scale of Intelligence-Revised (WASI-R) in a subgroup of participants to examine for associations of task performance with IQ.

Data acquisition

Imaging Echo planar images sensitive to BOLD signal changes were acquired on a 3.0 T General Electric™ whole body MR scanner during performance of the homophone task. We used the following scan parameters: flip angle 90 degrees, TE/TR 40/2000 ms, field of view 24 × 24 cm, 19 slices, slice thickness of 5 mm, gap 0 mm, and a 64 × 64 imaging matrix with in-plane resolution of 3.75 mm.

Data pre-processing Functional scans were analysed in Brain Voyager QX (BVQX), version 1.7 (Brain Innovation, Maastricht, The Netherlands). Data were pre-processed as follows: three-dimensional motion correction by trilinear interpolation. Data having greater than 2 mm translational movement or 2 mm rotational movement was excluded. Linear trend removal and a three cycles/time course high pass filter were used. Scans were transformed into Talairach coordinate space (Talairach, *et al*., 1988).

Intrasubject fMRI modelling Using a Talairach BA atlas, images for regional BOLD signal time course analysis were masked to include only left BA 9, 18, 21/22 and 45. This was accomplished in BVQX by loading the Talairach atlas Daemon as a BA voxel of interest file and creating a mask file using the BA regions (Lancaster, *et al*., 2000). Tasks were modelled as predictors in BVQX to screen data for activation in the regions of interest and to generate images for displaying activation. We used a false discovery rate (FDR) of 0.05 corrected for total voxels in the regions of interest to display the regional brain activation maps for Figure 2 (Genovese, *et al*., 2002). The time course was modelled using a two gamma hemodynamic response function with onset 0, time to peak 5 s, response undershoot ratio of 7, time to undershoot peak of 15 s, response dispersion 1 and undershoot dispersion 1. Because we did not plan a case/control comparison, we did not use a statistical cutoff to produce regional brain activation maps for analysis but instead analysed the percent BOLD signal change during task performance for each subject individually as the contrast of word encode minus pseudo-word encode and word recognition minus pseudo-word recognition. Using BVQX, average time course percent BOLD signal change was computed for each region in each subject as (BOLD signal during task − BOLD signal during baseline/BOLD signal during baseline) × 100. The regional percent BOLD signal change data were then entered into the correlation analyses (see below).

Statistical analysis

Outcome measures of percent BOLD signal change for each area of interest, number of correct responses, number of omission errors, number of commission errors and response time (for correct responses) for recognition were exported to SPSS for statistical analysis (SPSS for Windows version 15.0 software; SPSS Inc., Chicago, IL, USA). Continuous variables were analysed using Spearman's test. Results were considered statistically significant on two-tailed analysis if $P < 0.05$. Results presented are means and standard deviations unless otherwise specified.

Results

Demographics

All subjects were right-handed. Ten were men and six were women. One subject was black, one was Asian, one was Asian/Caucasian and all other participants were Caucasian. Age was 23.6 ± 2.7 years (range 20–29 years) and WASI verbal intelligence score was 60.2 ± 7.4 (range 50–71). WASI verbal IQ was within normal range and consistent with verbal IQ scores previously recorded in MDMA users and controls (Zakzanis, *et al*., 2002).

Drug exposure history

Lifetime drug use exposure as episodes and units is summarized in Table 1 as mean, standard deviation of the mean (SD) and minimum and maximum for subjects reporting exposure to a specific drug. In all, 12 of our total cohort of 16 polysubstance users used MDMA. All MDMA users also used cannabis and alcohol. Ten subjects used cocaine, of whom eight were MDMA users. Six subjects used methamphetamine, of whom five were MDMA users. But for three subjects who had used codeine, there was no reported use of opioids in our cohort. There were statistically significant correlations between lifetime episodes of MDMA use and lifetime episodes of cannabis use ($r_s = 0.752$, $P = 0.001$), cocaine ($r_s = 0.666$, $P = 0.005$), lysergic acid diethylamide (LSD) ($r_s = 0.686$, $P = 0.003$) and methamphetamine ($r_s = 0.750$, $P = 0.001$). There were no statistically significant correlations between lifetime episodes of MDMA and lifetime episodes of use of alcohol ($r_s = 0.094$, $P = 0.739$) or psilocybin ($r_s = 0.465$, $P = 0.069$).

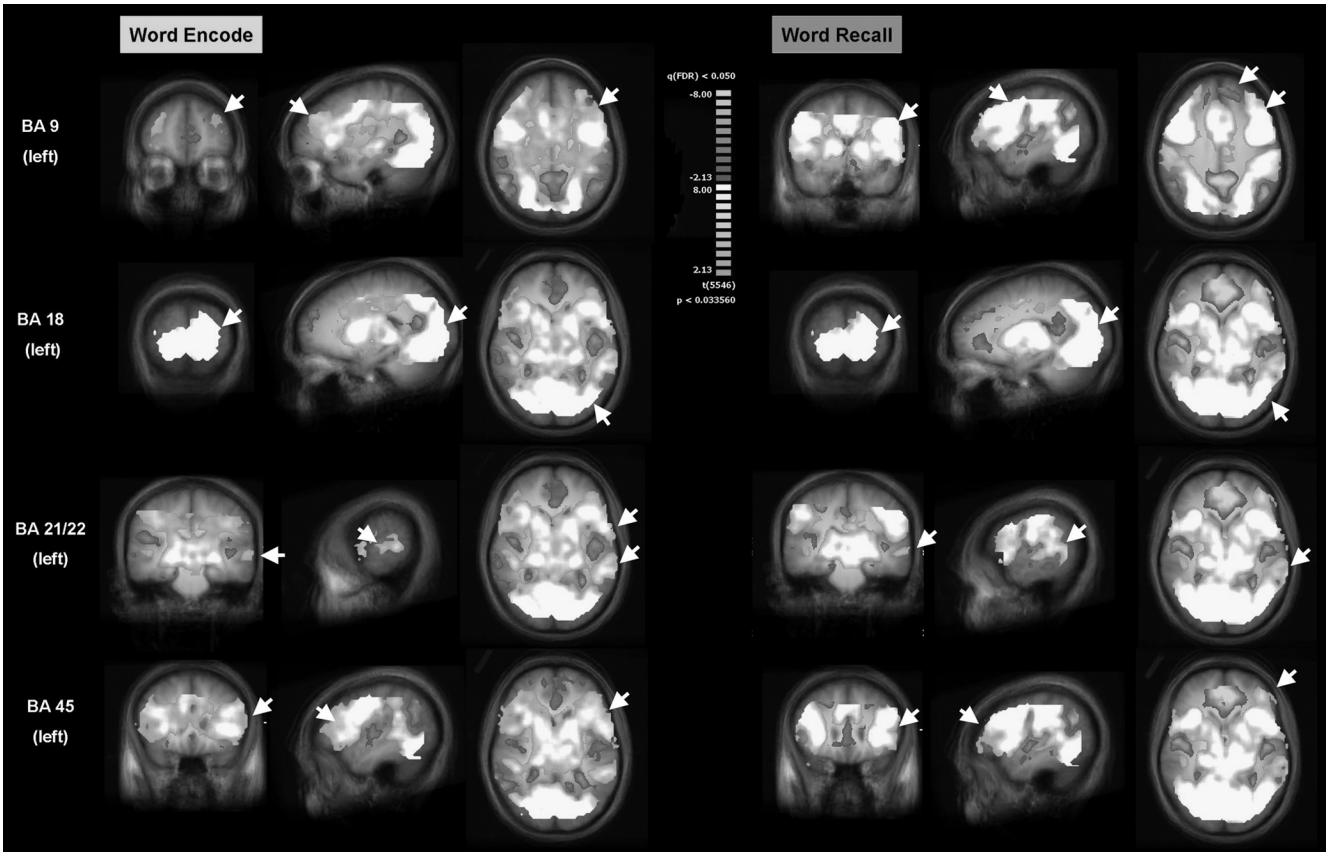

Figure 2 Activation to word encode and word recognition. Task-induced regional brain activation maps for word encode and word recognition. Maps were thresholded at a false discovery rate (FDR) of <0.05 (see methods for details). Rows show (top to bottom) regional brain activation to Brodmann area (BA) 9, 18, 21/22 and 45. White arrowheads point to BA in each brain view. For each condition (word encode or word recognition), activation maps are overlayed on a group average structural brain in (left to right) coronal, sagittal and axial sections. Right side of figure is left side of brain for coronal and axial sections. Right side is posterior for sagittal sections. Scale bar indicates *t* values ranging from −8.00 to +8.00. Warm colours are positive *t*, indicating increased activation with task activity. Cool colours are negative *t*, indicating decreased activation with task activity. All regions of interest used for analysis showed increased task-induced activation.

The length of abstinence from substance use before starting the study is shown in Table 2. All MDMA users were abstinent from MDMA for over 2 weeks. One subject in the cannabis cohort used 1 day before the study, whereas all other users reported no use for at least 4 days. Cocaine users had been abstinent for at least 16 days. Methamphetamine use was remote with reported last use at least 157 days ago.

Drug use and regional brain activation

Overall task effects As shown in Figure 2, the word encode and word recognition tasks produced robust and widespread regional brain activation at the selected statistical threshold (FDR = 0.05). More specifically, the task activated left BA 9, 18, 21/22 and 45 in both conditions. A similar pattern was seen for the pseudo-encode and pseudo-recall task (not shown). The analysis of percent BOLD signal change for semantic encode or recognition (defined as percent signal change during word task-percent signal change during pseudo-word task) showed that our task produced net positive activations for semantic encode and recognition in BA 9, 18 and 21/22. For BA 45, the task produced negative BOLD signal change for semantic encode and positive BOLD signal change for semantic recognition (Figure 3).

Semantic encode For the semantic encode task, there was no association between lifetime MDMA use and percent BOLD signal change for left BA 9, BA 18, BA 21/22 or BA 45 (Table 3—all correlations are calculated within the group exposed to a particular drug). For the other most commonly used drugs (alcohol, cannabis, methamphetamine, LSD and psilocybin), there were no significant associations between drug use and percent BOLD signal change during semantic encode for BA 18 or BA 21/22. For BA 9, there was a

Table 1 Lifetime substance use

Drug	*n*	Episodes use				Quantity use			
		Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum
MDMA (mg)	12	43.33	40.73	8	155	4607.08	4131.24	750.0	15 325
Alcohol (units)	15	548.73	751.75	4	2110	1761.60	2721.14	4.0	10 171
Cannabis (joints)	12	726.33	710.38	9	2000	2092.56	3725.10	8.0	12 904
Cocaine (mg)	9[a]	46.60	124.31	1	400	7.17	14.74	0.3	46
LSD (mcg)	9	24.89	21.77	4	65	5758.33	6765.29	295.0	18 700
Psilocybin (mg)	8	9.38	6.78	2	20	32.69	28.64	2.0	75
Meth (mg)	7	56.43	63.68	1	166	2468.57	2989.58	40.0	8110

MDMA, 3,4-methylenedioxymethamphetamine; LSD, lysergic acid diethylamide; Meth, methamphetamine.
[a]Data missing from one subject.

significant negative correlation between lifetime methamphetamine use and percent BOLD signal change ($r_s = -0.786$, $P = 0.036$). For BA 45, only lifetime episodes of alcohol use showed a significantly positive association with percent BOLD signal change ($r_s = 0.518$, $P = 0.048$).

Semantic recognition Within the MDMA-exposed group, there were statistically significant negative correlations between MDMA use and percent BOLD signal change in left BA 9, 18 and 21/22 but not BA 45 (Table 3). For BA 9, both lifetime episodes of MDMA use and lifetime milligrams of MDMA use showed a statistically significant inverse association with percent BOLD signal change. Only lifetime episodes of MDMA use was statistically significantly inversely associated with percent BOLD signal change in BA 18 and 21/22.

Within the cannabis-exposed cohort, there was a significant negative correlation between lifetime episodes of use and percent BOLD signal change in left BA 9 ($r_s = -0.580$, $P = 0.048$) and for lifetime joints used and percent BOLD signal change in left BA 45 ($r_s = -0.587$, $P = 0.045$). After controlling for cannabis use in the covariate analysis, lifetime episodes of MDMA use remained statistically significantly negatively associated with percent BOLD signal change in left BA 9 ($r_s = -0.680$, $P = 0.021$).

Within the cocaine-exposed group, cocaine exposure was inversely associated with percent BOLD signal change in left BA 9 and 18 during semantic recognition. This was true for both lifetime episodes of use (BA 9: $r_s = -0.851$, $P = 0.002$; BA 18: $r_s = -0.669$, $P = 0.035$) and lifetime grams of cocaine used (BA 9: $r_s = -0.683$, $P = 0.042$; BA 18: $r_s = -0.767$, $P = 0.019$). The negative correlation between lifetime episodes of MDMA use and BA 9 remained significant after controlling for the association of BA 9 with lifetime episodes of cocaine ($r_s = -0.669$, $P = 0.049$). After controlling for lifetime episodes of cocaine use, the negative correlation between lifetime episodes of MDMA and percent BOLD signal change in left BA 18 no longer achieved statistical significance ($r_s = -0.534$, $P = 0.139$).

For all other most commonly used drugs (cohorts with exposure to alcohol, methamphetamine, LSD or psilocybin), there was no statistically significant association between substance exposure and BOLD activation during semantic recognition.

Abstinence

There was a statistically significant negative correlation between days since last use of MDMA and activation in BA 18 for semantic recognition ($r_s = -0.700$, $P = 0.016$). There

Table 2 Abstinence from drug use

Drug	*n*	Mean days since last use	SD	Minimum	Maximum
MDMA	11	476.55	326.28	19	1132
Alcohol	16	32.19	68.87	1	274
Cannabis	12	75.33	136.17	1	385
Cocaine	10	234.4	283.87	16	764
LSD	8	897.63	772.31	110	2227
Psilocybin	7	718.71	649.71	60	1862
Meth	6	875.17	759.75	157	2224

MDMA, 3,4-methylenedioxymethamphetamine; LSD, lysergic acid diethylamide; Meth, methamphetamine.

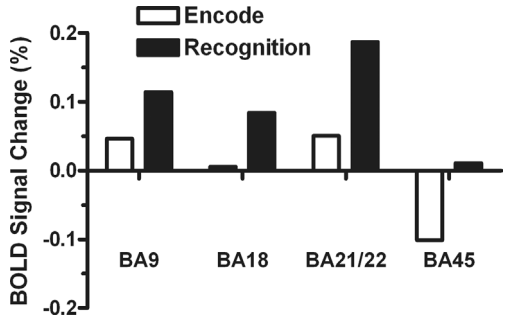

Figure 3 Regional semantic percent BOLD signal change. Bar chart indicating regional semantic percent BOLD signal change as the percent signal change in a region of interest for word encode or recognition minus pseudo-word encode or recognition.

were also statistically significant negative correlations between days since last use of cannabis and regional brain activation in BA 45 for semantic encode ($r_s = -0.715$, $P = 0.009$) and semantic recognition ($r_s = -0.753$, $P = 0.005$).

Drug use and task performance

For the subgroup of subjects having WASI verbal IQ scores, there was no statistically significant association of WASI score with lifetime episodes ($r_s = -0.186$, $P = 0.631$, $n = 9$) or milligrams ($r_s = -0.267$, $P = 0.488$, $n = 9$) of MDMA use, or between WASI score and regional brain activation in left BA 9, 18, 21/22 or 45 for semantic encoding or semantic recognition. Further, there was no association of WASI score with recognition task performance measures [percent correct responses, reaction time or accuracy (number of omission and commission errors) for both the word and pseudo-word portions of the task].

For the MDMA users, there was no significant association between lifetime episodes or milligrams of MDMA use and the recognition task performance measures (Table 4—all correlations are calculated within the group exposed to a particular drug).

For the cannabis-exposed group, there was a statistically significant negative correlation between lifetime cannabis exposure and reaction time on the word portion of the recognition task (Table 4). This was true for both episodes of use ($r_s = -0.767$, $P = 0.016$) and number of joints used ($r_s = -0.700$, $P = 0.036$). Further, there was a significant negative correlation between lifetime joints of cannabis and accuracy (number of commission errors) on the pseudo-word portion of the recognition task ($r_s = -0.691$, $P = 0.039$).

For psilocybin-exposed subjects, there was a significant positive association between lifetime grams of psilocybin used and percent correct responses on the pseudo-word portion of the recognition task ($r_s = 0.855$, $P = 0.014$).

Across the full cohort of subjects, there was no significant association between the recognition task performance measures and regional brain activation during word and pseudo-word recognition for left BA 9, 18, 21/22 or 45. Because we used a

Table 3 Brain activation and lifetime drug use correlations

Lifetime episodes	*n*	Semantic encode				Semantic recognition			
		BA 9	BA 18	BA 21/22	BA 45	BA 9	BA 18	BA 21/22	BA 45
		r	*r*	*r*	*r*	*r*	*r*	*r*	*r*
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*
MDMA	12	−0.028	−0.169	−0.070	−0.035	**−0.789**	**−0.606**	**−0.683**	−0.324
		0.931	0.599	0.828	0.913	**0.002**	**0.037**	**0.014**	0.304
Alcohol	15	0.379	0.250	−0.098	**0.518**	0.004	−0.250	−0.121	−0.214
		0.164	0.369	0.727	**0.048**	0.990	0.369	0.666	0.443
Cannabis	12	0.147	−0.189	0.217	0.308	**−0.580**	−0.531	−0.182	−0.483
		0.649	0.557	0.499	0.331	**0.048**	0.075	0.572	0.112
Cocaine	10	0.310	−0.170	0.103	0.413	**−0.851**	**−0.669**	−0.608	−0.571
		0.383	0.638	0.776	0.235	**0.002**	**0.035**	0.062	0.084
LSD	9	−0.183	−0.517	−0.067	−0.383	−0.083	0.300	0.167	−0.233
		0.637	0.154	0.865	0.308	0.831	0.433	0.668	0.546
Psilocybin	8	−0.263	−0.707	−0.563	−0.299	−0.299	0.311	0.192	−0.347
		0.528	0.050	0.146	0.471	0.471	0.453	0.649	0.399
Meth	7	**−0.786**	0.464	0.464	−0.571	−0.357	−0.464	−0.464	−0.214
		0.036	0.294	0.294	0.180	0.432	0.294	0.294	0.645

BA, Brodmann area; MDMA, 3,4-methylenedioxymethamphetamine; LSD, lysergic acid diethylamide; Meth, methamphetamine.
Bolded values indicate statistically significant ($P < 0.05$) Spearman's correlations (r) and corresponding P values (P) between lifetime episodes of drug use and regional brain activation for semantic encode and semantic recognition.

Table 4 Lifetime episodes of drug use and semantic task performance correlations

Lifetime episodes	*N*	Word				Pseudo-word			
		Percent correct	Reaction time (ms)	Omission (n)	Commission (n)	Percent correct	Reaction time (ms)	Omission (n)	Commission (n)
		r	*r*	*r*	*r*	*r*	*r*	*r*	*r*
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*
MDMA	9	0.051	−0.502	0.176	0.145	0.051	0.259	0.061	−0.184
		0.896	0.168	0.651	0.710	0.897	0.500	0.877	0.635
Alcohol	13	0.352	−0.352	−0.630	−0.271	−0.171	−0.203	0.356	−0.118
		0.239	0.239	0.838	0.370	0.576	0.505	0.233	0.701
Cannabis	9	0.458	**−0.767**	−0.096	−0.076	0.580	−0.333	−0.397	−0.411
		0.215	**0.016**	0.806	0.845	0.102	0.381	0.290	0.272
Cocaine	8	0.311	−0.452	0.061	−0.436	−0.132	−0.143	0.331	−0.358
		0.453	0.260	0.885	0.280	0.756	0.736	0.423	0.385
LSD	7	0.074	0.250	0.487	−0.436	0.309	−0.071	0.154	−0.468
		0.875	0.589	0.268	0.328	0.500	0.879	0.741	0.290
Psilocybin	7	0.150	−0.360	0.663	−0.422	0.587	−0.414	−0.156	−0.577
		0.749	0.939	0.104	0.346	0.166	0.355	0.739	0.175
Meth	6	−0.406	−0.486	0.213	0.493	0.143	0.371	−0.334	−0.031
		0.425	0.329	0.686	0.321	0.787	0.468	0.518	0.954

MDMA, 3,4-methylenedioxymethamphetamine; LSD, lysergic acid diethylamide; Meth, methamphetamine.
Bolded values indicate statistically significant ($P < 0.05$) Spearman's correlations (r) and corresponding P values (P) between lifetime episodes of drug use and semantic recognition task performance measures for semantic encode and semantic recognition.

block design for the fMRI task, we could not isolate individual trials to segregate brain activation by correct/incorrect trial.

Discussion

Interpretation of results

In this cohort of polysubstance users, lifetime MDMA exposure was statistically significantly associated with reduced brain activation during a semantic recognition task across multiple Brodmann regions (left BA 9, 18 and 21/22). MDMA use was not statistically significantly associated with regional brain activation during semantic encoding, and there were no clear patterns of effects from other drugs. The association of reduced regional brain activation in left BA 18 and 21/22 with MDMA use is consistent with Cowan, *et al.*'s previous report of reduced brain grey matter concentration in the same regions in a cohort of MDMA polydrug users (there was no overlap between participants in the previous and current report) (Cowan, *et al.*, 2003). The finding of reduced brain activation in left BA 18 and 21/22 may suggest a locus for brain regions mediating the well-established reduction in aspects of verbal memory in MDMA users. The finding of an MDMA effect in the recognition but not the encoding phase suggests that verbal memory may be impaired at the level of recognition and not encoding. This is consistent with our prediction that a structural reduction in brain grey matter would be associated with functionally altered regional brain activation during verbal semantic recognition.

In our cohort of polysubstance users with a history of moderate lifetime MDMA exposure (according to the criteria of Fox, *et al.*, 2001), lifetime episodes of MDMA and cocaine use were not associated with altered performance on the verbal recognition task (as measured by word recognition reaction time or number of omission and commission errors), whereas lifetime cannabis use was associated with shorter word recognition reaction time and worse accuracy (increased commission errors). This contrasts with previous studies that have found evidence of verbal memory impairment in MDMA users (Laws and Kokkalis, 2007; Reneman, *et al.*, 2001b; Schilt, *et al.*, 2007). Possible interpretations include lifetime MDMA exposure in our cohort being too low to result in significant impairment in task performance (Parrott, 2006; Reneman, *et al.*, 2001b), confounding by use of other substances, small sample size or neurocognitive deficits that our verbal recognition task lacked sensitivity to detect. The relationship between BOLD signal and task performance is difficult to predict. However, it is important to note that there was an inverse relationship between MDMA use and BOLD signal change for brain regions that are implicated in semantic processing.

Task

Our paradigm intended to distinguish brain activation attributable to semantic memory. It is important to recognize that task

limitations precluded complete isolation of semantic memory. As shown in Figure 3, differences in percent BOLD signal change for the semantic contrast were generally small. This may be due to the fact that much of the task-related activation was due to non-specific aspects of working memory or because the percent BOLD signal change was averaged over an entire BA, which may not have exact correspondence to the portion of the region of interest relevant to semantic processing. Working memory was significantly represented in our paradigm due to the relatively short duration of time (10 s) between presentation of the words and onset of the forced choice task (Linden, 2007). This is likely to have contributed to the activation observed in BA 9 and 45 in particular, which are both significantly associated with working memory function in addition to their roles in semantic recognition. It is also possible that several pseudo-words may have been adequately reminiscent of English words to result in limited semantic activation in some subjects during the pseudo-word portion of the task.

Polysubstance use

Lifetime cocaine exposure was associated with reduced regional brain activation in left BA 9 and 18, whereas lifetime cannabis use was associated with reduced brain activation in left BA 9 during semantic recognition. After controlling for lifetime exposure to cannabis and cocaine, the association of MDMA use with BA 9 activation remained significant. However, the association between MDMA exposure and brain activation in BA 18 was no longer significant on controlling for lifetime cocaine use.

Use of cocaine was reported in 8 of the 12 MDMA polysubstance users. Several studies have reported impaired delayed and short-term verbal memory in chronic cocaine users compared with controls (Ardila, *et al*., 1991; Mittenberg and Motta, 1993; Pace-Schott, *et al*., 2008). This may have confounded the association of lifetime MDMA use with reduced left BA 18 (occipital cortex) activation because Tomasi, *et al*. (2007) have reported increased BOLD activation in the occipital cortex of cocaine users during a working memory task. The association of cocaine exposure with increased brain activation in the occipital cortex contrasts with the present study's finding of the association of MDMA exposure with reduced brain activation in left BA 18. This suggests that MDMA exposure is likely to be a primary driver of the association for cocaine and MDMA with reduced regional brain activity in left BA 18.

A history of significant methamphetamine use was reported in 7 of the 12 MDMA users (56.4 ± 63.7 lifetime episodes of use). Use was typically remote (875.2 ± 759.8 days since last dose). There was a statistically significant negative correlation between lifetime episodes of methamphetamine use and percent BOLD signal change in left BA 9 for semantic encode ($r_s = -0.786$, $P = 0.036$) but not for the semantic recognition portion of the task. There were no associations between lifetime use of any other drugs and regional brain activation for semantic encoding. There were no correlations between methamphetamine use and task performance measures. Neuropsychological studies have shown deficits in explicit memory, attention and selective inhibition in methamphetamine users. Our findings are consistent with several functional studies showing reduced activation in brain regions involved in semantic and working memory processing in methamphetamine-dependent subjects. Paulus, *et al*. (2003) found reduced activation in parietal cortical regions (precuneus and post-central gyrus) in methamphetamine-dependent subjects participating in a decision-making task. The same group found that methamphetamine-dependent subjects showed reduced activation of dorsolateral prefrontal cortex (BA 9) during performance of a prediction task (Paulus, *et al*., 2002). Relapsed methamphetamine-dependent subjects also showed hypoperfusion in the left superior temporal gyrus (BA 21/22), a region involved in lexical-semantic processing (Paulus, *et al*., 2005).

Every MDMA polysubstance user in our cohort had a history of cannabis use, which has been commonly reported in MDMA users and represents a significant potential confounder (Gouzoulis-Mayfrank and Daumann, 2006; Parrott, 2006). The statistically significant negative correlations between days since last use of cannabis and regional brain activation in BA 45 for semantic encode and semantic recognition are consistent with literature supporting reduced subacute brain activation in recently abstinent cannabis users, particularly in the prefrontal cortex. Several neuropsychological studies have shown that cannabis use may have chronic negative effects on short-term memory, including working and episodic memory (Bolla, *et al*., 2002; Pope Jr., *et al*., 2001; Solowij, *et al*., 2002; Yucel, *et al*., 2008). Herning, *et al*. (2005) found increased resistance of cerebral vessels during cannabis withdrawal. Several functional studies in recently abstinent chronic cannabis users have shown decreased regional cerebral blood flow in prefrontal cortex at rest (Sneider, *et al*., 2008) and during performance of a verbal memory task (Block, *et al*., 2002). Using PET, Block, *et al*. (2002) found a corresponding decrease in brain activation in memory-associated regions during performance of a word recall task in recently abstinent cannabis users. Conversely, some studies have found evidence of increased activation (Chang, *et al*., 2006). Our analysis showed that the relationship between lifetime MDMA exposure and reduced activation in BA 9 during semantic recognition remained significant when controlling for cannabis exposure. Cannabis use did not show as widespread or consistent a negative correlation with brain activation as MDMA use. This suggests that the observed statistical findings may be more determined by the strong correlation of MDMA use with cannabis use rather than with specific effects of cannabis.

Neocortex

Brain regions that have been associated with semantic processing include left temporal cortex (BA 21/22) and left parietal cortex (Friedman, *et al*., 1998). BA 18 is located in occipital cortex and is involved in visual learning involving name-face recognition (Herholz, *et al*., 2001). Left BA 21 is involved in semantic memory retrieval and higher level processing of

meaning (Booth, *et al*., 2002; Chou, *et al*., 2006; Lee, *et al*., 2002). Left BA 22, which was confluently activated with BA 21 during our fMRI paradigm, corresponds to left superior temporal gyrus. It is involved in converting sensory input into recognized word forms (Petersen, *et al*., 1988), which was an integral requirement in our task.

Left BA 45 is activated during tasks involving semantic memory retrieval (Booth, *et al*., 2002; Lee, *et al*., 2002), semantic fluency (Amunts, *et al*., 2004) and verbal working memory (Wager, *et al*., 2005), and its activation is greater during more demanding semantic tasks (Chou, *et al*., 2006). However, the role of BA 45 in these tasks may be related to a more general executive control process rather than specific to semantic processing. Although we did not find a relationship between MDMA exposure and activation in left BA 45, there was a significant relationship between lifetime joints of cannabis use and reduced brain activation in this region.

Left BA 9 corresponds to the dorsolateral prefrontal cortical region of the frontal lobe (Fitzgerald, *et al*., 2006). It is associated with central executive function including attentional selection, impulsivity, decision making, organization and monitoring of memory stimuli (Baxter, *et al*., 2008; Robbins, 2005), and it also shows significant involvement in working memory function (Funahashi, 2006; Babiloni, *et al*., 2005; Wager, *et al*., 2005). This region was strongly activated by our fMRI task. During this study, lifetime use of MDMA, cannabis and cocaine were each associated with reduced brain activation in this region, and the association with MDMA exposure remained statistically significant when controlling for cannabis and cocaine exposure. In contrast to our findings of reduced left BA 9 activation, Moeller, *et al*. (2004) found greater BOLD activation in prefrontal cortex in MDMA users compared with controls during a delayed working memory task. This difference may be secondary to the different tasks used in the two studies or to other experimental or cohort effects.

Implications of altered regional brain activation

The association between the degree of MDMA exposure and reduced activation in left BA 9, 18 and 21/22 permits several possible explanations regarding the neural origins of this finding. Although the finding of a consistent exposure-response relationship involving multiple regions involved in semantic recognition is suggestive of causation, there are several other potential interpretations of this finding. First, because there were associations between regional brain activation and exposure to MDMA, cannabis, cocaine and methamphetamine, it is possible that the current results are related to pre-existing brain differences that predispose to increased polydrug use or increased likelihood for MDMA consumption. However, the correlation between degree of drug use and outcomes was most consistent in the regions of interest for MDMA effects, which does not support a predisposition to drug use in general. Second, the regional brain activation could be reflective of a general effect of drug exposure. However, the fact that MDMA effects remained significant after controlling for cannabis and cocaine use in left BA 9 suggests that there may be a specific effect of MDMA. Third, other unknown factors not assayed or controlled for in this study could independently correlate with MDMA use and with regional brain activation. Fourth, although altered regional brain activation suggests that neural function is altered, the direction of altered brain activation (i.e., increased or decreased) does not permit conclusions regarding the pathological implications of the observed finding. Namely, both increases and decreases in regional brain activation have been associated with pathological processes (Bondi, *et al*., 2005). Present evidence suggests that activation in a brain region is most strongly determined by synaptic input to a brain region and secondarily by local synaptic processing in the brain region, but not by neuronal firing in the activated region (Rauch, *et al*., 2008). Other neurophysiological events may be largely undetectable by the BOLD method except as a downstream consequence of their effects on synaptic transmission (Logothetis and Pfeuffer, 2004). This suggests that reduced activation in a brain region may be more dependent on alterations in synaptic inputs to that region rather than a function of structural or functional alterations in the region of study. Because this study was primarily aimed at probing brain activation, we did not administer neuropsychological testing of verbal memory encoding and recognition separately from the tasks performed in the scanner. However, we did obtain verbal IQ assessments (WASI) and reaction time and accuracy measures for the recognition phase of the task. WASI scores did not show a correlation with MDMA exposure or performance on the verbal recognition task.

Neural basis of the observed findings

Findings from basic science investigations of MDMA effects provide a framework for interpreting the current results. Cowan, *et al*. (2008) have previously outlined a cortical model or framework for interpreting neuroimaging studies in human MDMA users. Essential features of the model relevant to the current report are 1) MDMA exposure is associated with persisting changes in serotonergic neurotransmission, 2) changes in serotonergic neurotransmission may be evident in loss of coupling between serotonin and brain neurotrophic factors (resulting in regional brain grey matter shrinkage) and 3) changes in serotonergic neurotransmission may be evident in altered cell-cell synaptic signalling irrespective of structural neuronal changes. As such, structural or functional effects of reduced 5-HT neurotransmission may contribute to the observed findings.

Initial studies of MDMA administration in animals suggested that MDMA produced a fine-diameter axotomy of 5-HT neurons with sparing of brainstem cell bodies (Green, *et al*., 2003; Kish, 2002). More recent animal studies have questioned this finding, suggesting that at doses potentially closely mimicking those of human recreational users, MDMA does not produce axotomy and loss of 5-HT markers does not necessarily indicate axotomy (Fantegrossi, *et al*., 2004; Wang,

et al., 2004; Wang, *et al*., 2005). One widely studied marker for 5-HT system integrity is the serotonin reuptake transporter (5-HTT). The 5-HTT is present on serotonergic axons and is the primary molecular structure controlling the duration of post-release 5-HT signalling. A reduction in 5-HTT levels following MDMA exposure is consistent either with serotonergic axotomy or with downregulation of transporter expression. Studies of 5-HTT binding have commonly shown reductions in 5-HTT levels in human MDMA users with some studies suggesting gender effects and others suggesting potential transporter recovery with increased duration of MDMA abstinence (de Win, *et al*., 2004; McCann, *et al*., 1998; McCann, *et al*., 2005; McCann, *et al*., 2008; Reneman, *et al*., 2001a; Semple, *et al*., 1999; Buchert, *et al*., 2004; Thomasius, *et al*., 2003). Additional study is needed before the specific time period and degree of recovery is fully clarified. A study examining 5-HT2A receptor expression in human MDMA users reported upregulation of these postsynaptic receptors in abstinent users, which is consistent with, but not confirmatory of, long-lasting reductions in the concentration of presynaptic 5-HT (Reneman, *et al*., 2002).

5-HT depletion by MDMA could play an important role in the verbal memory deficits observed in human MDMA users and the reduced activation in left BA 9, 18 and 21/22 observed in this study. Using tryptophan depletion to acutely lower 5-HT levels, Allen, *et al*. found increased BOLD activation in the left posterior cingulate cortex and reduced activation in the superior frontal gyrus and left precuneus during performance of a 2-back verbal working memory task. This suggested that 5-HT is involved in modulation of pre-frontal engagement during verbal working memory functioning (Allen, *et al*., 2006). Using fMRI to study subjects taking a serotonin selective reuptake inhibitor (SSRI) during performance of an N-back working memory task, Rose, *et al*. (2006) found increased left inferior frontal gyrus (BA 45) activation. Although it is tempting to speculate that the latter study supports the association of a 5-HT rich state with increased BOLD signal in a brain region involved in verbal working memory, caution must be used in extrapolating these findings to the effects of MDMA on the serotonin system and verbal memory. Of note, BA 45 was the one area studied where we found no statistically significant association of MDMA use with task-evoked BOLD signal. Although tryptophan depletion represents an acute state of 5-HT depletion, MDMA use is associated with more chronic serotonergic changes and possible adaptive neuromodulatory responses. Secondly, SSRIs are not completely selective in their effects on the serotonin system and their chronic effects are likely to induce significant adaptive changes (Butler and Meegan, 2008). Clearly 5-HT has a complicated role in memory function (Schmitt, *et al*., 2006).

Limitations

Several important limitations to this study restrict our ability to conclude whether or not MDMA-associated neurotoxicity is responsible for the observed findings. First, the cross-sectional correlational design of the study does not permit conclusions regarding cause and effect. It is possible that genetic or other influences predisposing to MDMA polydrug use may also be associated with pre-existing brain changes. Second, it is possible that a larger sample size may have resulted in some non-significant trends reaching statistical significance. Further, because regional semantic percent BOLD signal change was generally small (Figure 3), this could have resulted in failure to find correlations between MDMA use and activation in some cases. Third, the degree of polydrug exposure seen worldwide in MDMA users precludes the ability to recruit a well-matched control group (van Reekum, *et al*., 2001). MDMA users tend to use more of every class of drug than do their non-MDMA using peers (de Almeida and Silva, 2003; Scholey, *et al*., 2004; Wish, *et al*., 2006), and polydrug use can particularly confound case control designs when a class of drug is strongly correlated with MDMA use or when associated with outcome measures. In a functional neuroimaging report of visual system activation, MDMA users and controls did not differ in degree of mean regional brain activation while MDMA use (but not other drugs) was positively correlated with activation measures (Cowan, *et al*., 2007). This suggested that other drug exposure may have affected the mean activation in the MDMA group. Therefore, within group designs assaying for correlations between the degree of MDMA exposure and outcome variables may have specific utility in the study of brain function in human MDMA-exposed cohorts. Fourth, in addition to testing our primary hypothesis, we performed several exploratory correlational analyses examining the association of drug exposure and percent BOLD signal change in four brain regions across two tasks. Because increases or decreases in percent BOLD signal change in a brain region may be associated with functional decrements, this hypothesis required a two-tailed test for significance. Because of the exploratory nature of the additional comparisons, we did not use correction for multiple independent statistical comparisons. As such, it is possible that some of our findings were due to type I error related to multiple comparisons. Fifth, because VBM studies of the type previously reported require a much larger cohort than presented here, we do not yet know whether the current group of MDMA users show regional brain grey matter changes in the regions previously reported by Cowan, *et al*. (2003). Our preliminary analysis (unpublished observation) of a larger cohort that includes members from this study is not consistent with structural effects in these regions. Data collection for a VBM study that will include the current cohort is ongoing and larger longitudinal studies, such as those of the Netherlands XTC project (de Win, *et al*., 2005), are necessary to more fully address these confounds.

Because we used an *a priori* hypothesis-based region of interest analysis, we did not explore additional brain regions potentially involved in aspects of memory encoding and retrieval. Others have speculated that altered hippocampal structure and function may be related to impaired memory

encoding in MDMA users (Daumann, *et al*., 2005; Jacobsen, *et al*., 2004). Specific tasks designed to probe hippocampal function in MDMA users are needed to address this topic. As reviewed (Cowan, 2007), there is presently little overlap between various imaging modalities and their outcomes with regard to studies of MDMA polydrug users.

Conclusions

Using an fMRI semantic memory paradigm in a cross section of polysubstance users enriched for MDMA use, this study found an association between lifetime MDMA use and reduced BOLD activation in left BA 9, 18 and 21/22 but not 45 during semantic recognition. There was no significant association between MDMA exposure and performance time or accuracy on the semantic recognition task.

The finding of reduced brain activation in left BA 9, 18 and 21/22 suggests a potential locus for brain regions mediating the well-established reduction in aspects of verbal memory in MDMA users. The association of reduced brain activation with lifetime MDMA use is suggestive of a possible causal role for MDMA in effecting functional changes in brain regions involved in verbal memory processing. Further investigation is required to replicate and elaborate on these preliminary findings.

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