Supporting Online Material for

Abnormal Brain Structure Implicated in Stimulant Drug Addiction

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Materials and Methods

Study sample

Participants were recruited by advertisements and from treatment services in the East Anglia region of the UK. All recruits were psychiatrically evaluated using the Structured Clinical Interview for DSM-IV. The sibling pairs were enrolled if three conditions were met: 1) same biological parents, 2) one sibling satisfied the DSM-IV-TR criteria for cocaine or amphetamine dependence, and 3) the other sibling had no personal history of substance dependence (except nicotine). Exclusion criteria, applied for all groups, were a lifetime history of a psychotic disorder, a history of a neurological illness, a neurodevelopmental disorder, or a traumatic head injury. Control participants could not have a personal or family history of drug/alcohol dependence. All participants had to be 18–55 years old and able to read and write in English. The rationale for focusing on stimulant drugs was based on an assumed high heritability of stimulant dependence. The protocol was approved by the Cambridge Research Ethics Committee (REC08/H0308/310; PI: KD Ersche) and written informed consent was obtained from all participants prior to study enrolment. All participants performed the stop-signal task after the brain scans.

Of the 154 participants who enrolled, 150 completed the study forming the following three groups of 50 individuals: drug-dependent individuals, their biological siblings and unrelated healthy volunteers. MRI brain scans for three drug-dependent individuals and one sibling were unavailable, leaving the sample as follows: drug-dependent individuals (n = 47), siblings (n = 49), healthy volunteers (n = 50). The sibling pairs had a shared familial environment during childhood, which in most cases lasted into adolescence (Table S1). The three groups were relatively well matched in terms of age, pre-morbid intelligence and levels of education; but differed with regard to gender because the majority of drug-dependent individuals were male.

All drug users met the DSM-IV-TR criteria for stimulant dependence (94% cocaine and 6% amphetamines). On average, they had been using stimulants for 16.3 years (± 7.6 SD), starting at the age of 16.5 years (± 2.8 SD). Except for three samples, the urine provided by the drug users tested positive for stimulant drugs. The majority of the drug users also met criteria for dependence on another substance (55% opiates, 26% alcohol, and 9% cannabis). The drug-taking experiences in both the siblings and control volunteers were minor, as reflected by very low scores on the Drug Abuse Screening Test and Alcohol Use Disorders Identification Test (Table S1). All urine screens provided by siblings and healthy volunteers were drug negative.

The positive urine screens for stimulants in our drug-dependent sample confirmed that these individuals were indeed current users. We contemplated asking stimulant-dependent individuals to withdraw before testing but prior evidence suggests that acute

withdrawal can itself have a deleterious effect on performance (*31*, *32*). We therefore preferred to screen clinically for acute intoxication sufficient to incapacitate participants. Moreover, Garavan *et al*. (*33*) have recently shown that response inhibition performance improves in stimulant-dependent individuals under the influence of cocaine, suggesting that the observed impairments in our sample of stimulant-dependent individuals are, if anything, more likely to be mitigated than exaggerated by the fact that they had recently consumed stimulant drugs.

We did not breathalyse participants immediately prior to testing but they certainly did not consume alcohol during the testing and assessment session, which took place approximately 4 hours prior to scanning. We are not aware of evidence showing that brain structure, as measured by MRI, changes acutely following consumption of alcohol. Chronic alcohol use is indeed associated with changes in brain structure; but in our sample there was no significant difference in drinking habits between the siblings and the control volunteers, as assessed by the AUDIT questionnaire ($t_{70.9}$=-0.72, P=0.472; Table S1). In other words, it seems unlikely that the endophenotypic profile of brain abnormality (Fig. S1) can be attributed to differences in alcohol exposure.

Stop-Signal task

All participants performed the stop-signal task as part of a larger neuropsychological test battery, as reported elsewhere. Participants were asked to make speedy responses on Go-trials (i.e. left button-press in response to a left-pointing arrow; right button-press in response to a right-pointing arrow), but to withhold responding on Stop-trials, which were intermittently signaled by a 300 Hz tone. Stopping was made difficult by the preponderance of Go-trials (75%) and the manipulation of the timing of the stop-signal by means of a tracking algorithm, allowing the estimation of the *stop-signal reaction time* (SSRT). The calculation of SSRT is based on the assumption that the motor responses to Go- and Stop-trials are independent. All participants performed five blocks of 64 trials each and received visual feedback after each block for the average reaction time for a correct response to Go-trials and the number of discrimination errors (i.e. incorrect response on Go-trials). Participants did not receive feedback in terms of successful or failed inhibition in response to Stop-trials, but they were urged to do their best to stop, while continuing to respond as fast as possible on Go-trials.

Statistical analysis

Demographic and behavioral data were analyzed using the Statistical Package for Social Sciences (SPSS, version 19). Analyses of co-variance (ANOVA) were used to explore group differences in demographics and stop-signal performance; gender was included as a covariate in all analyses to control for the significant group differences of gender. For post-hoc comparisons, Bonferroni correction was applied.

Acquisition of the neuroimaging data

All participants underwent magnetic resonance (MR) brain scans at the Wolfson Brain Imaging Centre, University of Cambridge, UK, using a Siemens TIM Trio 3T system. T1-weighted MR scans were acquired using a magnetization-prepared rapid acquisition gradient-echo (MPRAGE) sequence (176 slices of 1 mm thickness, TR = 2300 ms, TE = 2.98 ms, TI = 900 ms, flip angle = 9°, FOV= 240 x 256). Whole brain diffusion weighted EPI scans (63 slices of 2 mm thickness, TR = 7800 ms, TE = 90 ms, FOV = 192 x 192 mm, 96 x 96 in-plane matrix) were also acquired using 63 diffusion directions and b = 1000 s/mm^2. One b = 0 s/mm^2 scan was also acquired. All MR images were screened for abnormal radiological appearance by a specialist in neuroradiology.

MRI data processing and analyses

Gray matter volume maps of 146 participants (50 controls, 47 drug users and 49 siblings) were analyzed using FSLVBM (http://www.fmrib.ox.ac.uk/fsl/fslvbm/index.html, Version 4.1). Non-brain tissues were removed using the brain extraction tool of FSL (BET) and tissue-type segmentation was preformed using FAST. The resulting gray matter partial volume images were aligned to MNI-152 standard space using the affine registration tool FLIRT, followed by nonlinear registration using FNIRT, which uses a *b*-spline representation of the registration warp field. A study-specific gray matter template was made to which the native gray matter images were then nonlinearly re-registered. To correct for local expansion or contraction, the registered partial-volume images were modulated by division with the Jacobian warp field. The modulated segmented images were then smoothed with an isotropic Gaussian kernel with full width half maximum (FWHM) = 2.3 mm. The group comparisons of the gray matter maps were performed using CamBA software for permutation testing (*34*), version 2.3.0 (http://www-bmu.psychiatry.cam.ac.uk/software/) and thresholded at cluster-level statistics of $\eta = 1$ error clusters per image, with equivalent P-values: controls ≅ siblings, $P = 9 \times 10^{-4}$; and controls ≅ drug users, $P = 8 \times 10^{-4}$. In the first comparison, healthy volunteers versus siblings, a total of four clusters was identified, and in the second comparison, healthy volunteers versus drug users, a total of eight clusters was found. All voxels surviving the intersection of these distinct clusters were located within three anatomically distinct regions. Separate group comparisons in the three groups limited to either male or female gender did not change the results.

DTI data processing and analyses

Because one drug user was unable to tolerate a full scanning session, white matter volume maps of 145 participants (50 controls, 46 drug users and 49 siblings) were analyzed using the Diffusion Toolbox, implemented in FSL (www.fmrib.ox.ac.uk/fsl/fdt/) software. The 63 diffusion-weighted volumes were corrected for eddy currents and head motion by an affine transformation to the first non-diffusion volume (b = 0). For voxelwise statistical analysis of the FA data, tract-based spatial statistics (TBSS; www.fmrib.ox.ac.uk/fsl/tbss/) were used (*35*). Each FA map was up-sampled to 1 x 1 x 1 mm to form a mean FA image, which was thinned to create a

mean FA skeleton. The skeleton was thresholded at 0.2 to preserve only the clearest tracts. Each participant's FA map was aligned non-linearly to the FMRIB58_FA template using the default FA_2_FMRIB58_1mm.cnf configuration options. The aligned FA maps were then projected onto the skeleton, skeletonised, and the resulting data was entered into a voxelwise cross-subject statistical model. All FA-maps were visually inspected to identify any gross inaccuracies and to confirm an appropriate fit to the FA template.

A voxelwise three-group permutation F-test and two-group t-tests were performed using the randomise randomisation method with threshold-free cluster enhancement and 5000 permutations (*16*). Maps of significant FA clusters were corrected for multiple comparisons and thresholded at $P < 0.05$. Mean thresholded FA skeleton values were imported into SPSS for post-hoc comparison. Values for mean diffusivity (MD) were also calculated within each significant cluster. Hammer's probabilistic atlas (*19*) was used to generate regions of interest for the inferior frontal gyrus (IFG) and the pre-supplementary motor area (pre-SMA). The pre-SMA included the superior frontal gyrus at $0 < y < 20$ mm. Mean values of FA for IFG and pre-SMA in each hemisphere were imported into SPSS for post-hoc tests and correlational analysis.

Assessment of familiality

The variance of the within pair differences was calculated for each key measure: σ[drug user-sibling pair] = $\sum(u_j - \bar{u})^2 / n$ where u_j is the observed within-pair difference of the measure for the j^{th} pair of participants, and $\bar{u}$ is the mean within-pair difference and n is the total number of pairs (*36*). Then new pairs were randomly assigned, so that each drug-dependent individual was randomly assigned a sibling with whom they were not related. The variance of the within-pair difference in the permuted or randomized sibling pair was calculated after each random re-pairing and this process was repeated 100,000 times to sample the permutation distribution of σ[drug user-sibling pair] under the null hypothesis that the observed variance within-pair differences was not determined by the familial relatedness of the observed pairs. On the alternative hypothesis that the observed variance would be small, this was compared to the 5000^{th} value of the permutation distribution for a one-tailed test of the null hypothesis with $P < 0.05$.

Effects of tobacco smoking on gray and white matter

There were tobacco smokers in all three groups (12 % controls, 55% siblings, 94% drug users), and in the literature, tobacco smoking has been associated with gray matter changes in the prefrontal cortex, cingulate gyrus and anterior insula (Table S3). However, these are not the same areas of gray matter abnormality as we identified in the non-dependent siblings (Fig. S1). Our data indicates that the putamen, amygdala/hippocampus and the superior temporal gyrus/posterior insula are vulnerability markers for developing drug dependence. Thus, a detailed anatomical comparison of the areas implicated by tobacco smoking and the areas of abnormality in non-dependent siblings indicates that

environmental exposure to tobacco is unlikely to explain the pattern of gray matter abnormalities identified as an endophenotype for stimulant dependence.

We have carried out an additional analysis using only data acquired from non-smoking controls and siblings. As shown in Fig. S1, the profile of gray matter abnormality in amygdala, putamen and postcentral gyrus remained statistically significant even when all controls and siblings with any history of tobacco smoking were excluded from the analysis. This result shows that the differences in siblings we have attributed to genetic risk for stimulant dependence might instead be attributable to environmental exposure to tobacco.

Finally, we note that we were not surprised to see higher smoking rates in the siblings compared with the control volunteers because tobacco smoking is a form of drug dependency and, therefore, higher rates of tobacco smoking are expected in individuals at greater than normal genetic risk of drug dependence. For example, twin studies have shown that at least 30% of the variance tobacco smoking behaviour can be attributed to genetic factors (*37*). These and other data suggest that the higher rates of tobacco smoking seen in our sample of siblings may represent another aspect of their genetic risk for stimulant dependence rather than a confounding environmental cause of differences in their brain anatomy compared to healthy volunteers.

Fig. S1.

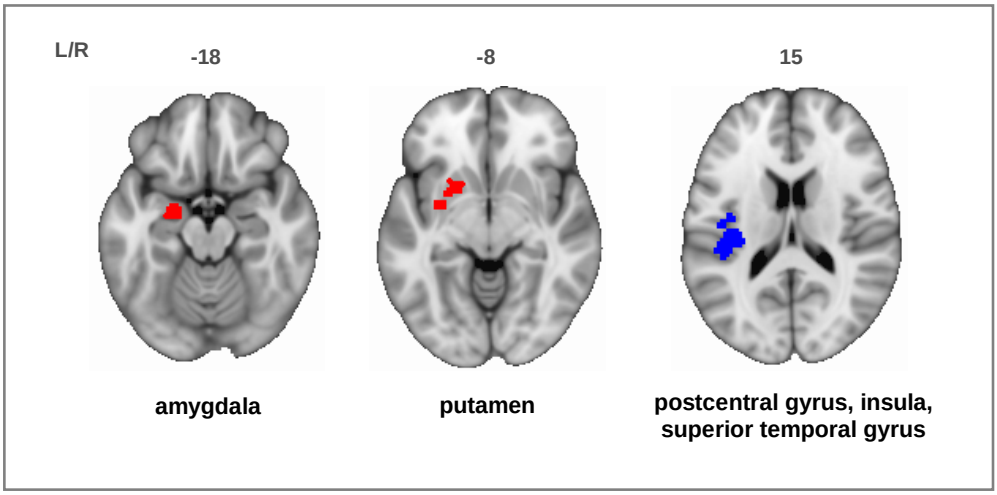

Fig. S1. Significant differences in gray matter density between non-smoking controls and non-smoking siblings were identified in the same brain regions that demonstrated an endophenotypic profile of abnormality in the full sample (smokers included in both groups; as in Fig. 2C). Left side of the brain is shown on the left side of each slice; the numbers denote z-coordinates for each slice in standard stereotactic space.

Fig. S2.

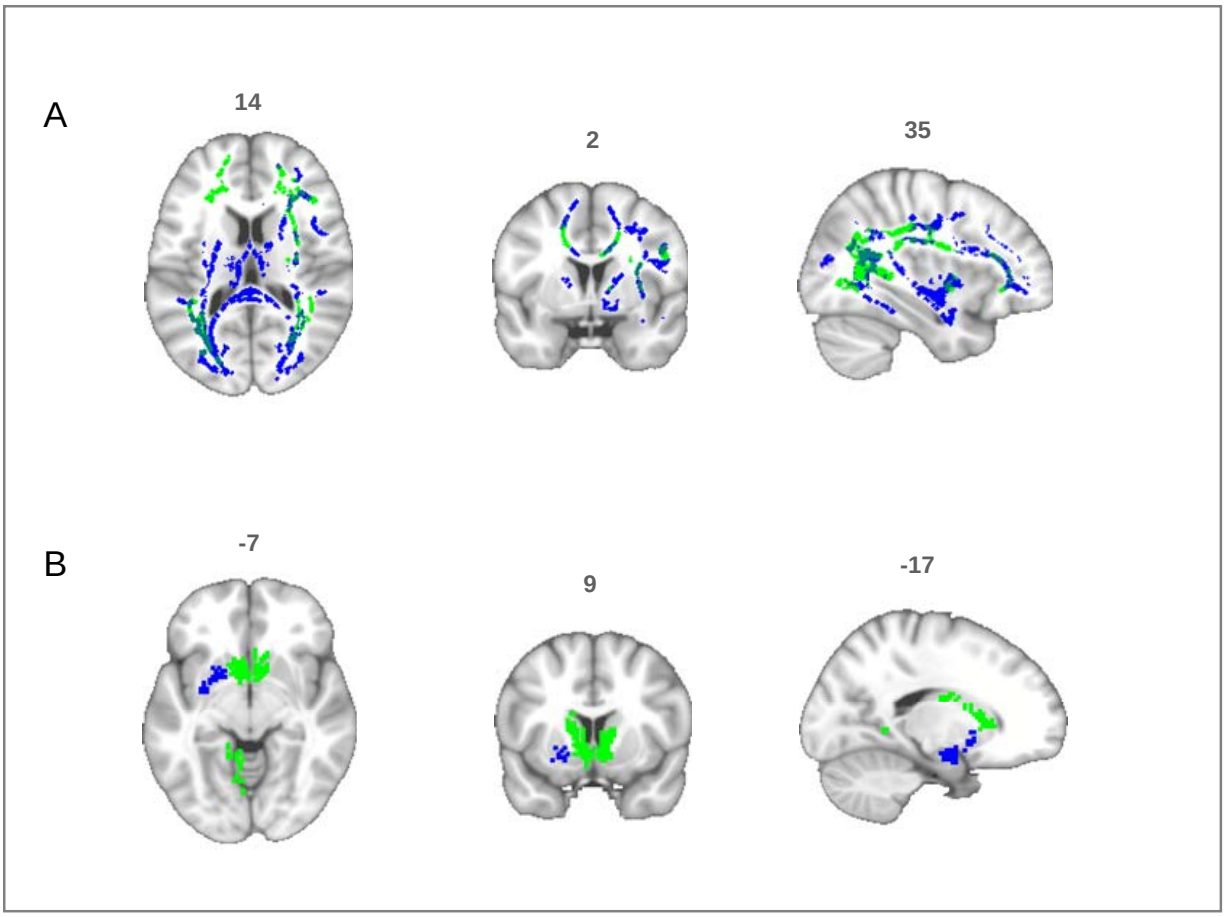

Fig. S2. (**A**) The FA endophenotype effect (drug users < siblings < controls) is colored in blue. Regions where FA is negatively correlated with the years of stimulant abuse are overlaid in green. This shows that duration of drug exposure has effects on white matter organization but these are less extensive anatomically than the effects of familial risk for stimulant dependence. (**B**) The endophenotype effect in the left amygdala and putamen (drug users = siblings > controls) is colored in blue. Gray matter regions that are negatively correlated with the duration of stimulant abuse are colored in green. These abnormalities associated with stimulant drug exposure are distinctly different from the endophenotypic effects.

Table S1. Demographic data in stimulant-dependent individuals, their biological siblings who do not have a history of drug dependence, and unrelated healthy control volunteers

	Healthy unrelated volunteers (*n*=50) Mean	Std.	**Non-dependent siblings (*n*=49)** Mean	Std.	**Stimulant-depend individuals (*n*=47)** Mean	Std.	***F* or χ^2**	***P***
Gender (% male)	64%		51%		92%		19.0	<0.001
Age (years)	32.8	(±8.9)	32.6	(±8.4)	34.5	(±7.4)	0.4	0.702
Verbal intelligence (NART*)	112.3	(±8.2)	108.9	(±8.9)	110.3	(±7.4)	2.0	0.143
Formal education (years)	12.6	(±1.9)	12.1	(±2.0)	11.6	(±1.7)	2.83	0.062
Current tobacco smokers	12%		55%		94%		71.8	<0.001
Same upbringing until age 10 years	--		90%		92%		Fisher exact	1.000
Same upbringing until age 15 years	--		75%		89%			0.107
Drug-taking experience (DAST-20†)	0.0	(±0.0)	0.5	(±1.1)	--		12.6	0.001
Alcohol consumption (AUDIT‡)	3.3	(±2.3)	3.9	(±4.5)	11.4	(±11.4)	13.4	<0.001
Stop-Signal P(stop)	55.0	(±3.4)	56.0	(±3.1)	56.8	(±5.1)	2.6	0.079
Stop-Signal reaction time SSRT	238.9	(±45.0)	276.7	(±55.5)	280.8	(±61.9)	8.9	<0.001

* NART: National English Reading Test

† DAST-20: A quantitative index of whether a person's drug use is harmful or not. Cut off score for harmful use: > 5.0

‡ AUDIT: A quantitative index of whether a person's alcohol consumption is harmful. Cut off score for harmful use: > 8.0

Table S2. Measures of white matter organization: mean fractional anisotropy and mean diffusivity ($x10^{-6}$ mm^2 s^{-1}) for clustered voxels that demonstrated significant differences between groups on whole brain mapping, and for regions of interest (inferior frontal gyrus and pre-SMA) defined by the prior criteria of the MNI Hammers atlas (S7). Clusters, anatomically labeled using the MNI Hammers atlas.

		Cluster	Voxels	Healthy Control volunteers	Non-drug abusing Siblings	Stimulant-dependent individuals
			number	mean	mean	mean
White Matter	Fractional anisotropy	Whole skeleton	24,934	0.55 (±0.02)	0.53 (±0.02)	0.52 (±0.02)
		Inferior frontal gyrus L	0	N/A	N/A	N/A
		Inferior frontal gyrus R	317	0.51 (±0.03)	0.49 (±0.03)	0.48 (±0.03)
		Pre-SMA L	156	0.61 (±0.03)	0.59 (±0.03)	0.58 (±0.05)
		Pre-SMA R	496	0.58 (±0.03)	0.57 (±0.03)	0.55 (±0.03)
	Mean diffusivity	Whole skeleton	24,934	710 (±23)	712 (±21)	730 (±25)
		Inferior frontal gyrus L	0	N/A	N/A	N/A
		Inferior frontal gyrus R	317	659 (±35)	656(±36)	668 (±38)
		Pre-SMA L	156	668 (±43)	684 (±43)	686 (±43)
		Pre-SMA R	496	687 (±40)	686 (±34)	698 (±31)
			mm^3	mean	mean	mean
Gray	Volume	Amygdala L	984	0.76 (±0.07)	0.81 (±0.07)	0.83 (±0.07)
		Putamen L	560	0.34 (±0.05)	0.38 (±0.06)	0.38 (±0.05)
		Postcentral gyrus L	1,152	0.89 (±0.12)	0.75 (±0.11)	0.74 (±0.11)

Table S3. Anatomical coordinates for all of the brain regions identified as abnormal in tobacco smokers in the several prior studies. None of these regions were identified as a candidate endophenotype for stimulant dependence in our data.

Our findings	**Candidate endophenotypes of stimulant dependence**	**X**	**Y**	**Z**
	Amygdala/hippocampus	–22	–6	–16
	Putamen	–24	2	–12
	Superior temporal gyrus / posterior insula	–36	–32	20
Published articles:	**Region associated with tobacco smoking**	**X**	**Y**	**Z**
Almeida *et al*. (2008) (*38*)	Posterior cingulate cortex (bilateral)	coordinates were not available		
	Precuneus (bilateral)			
	Frontal cortex (bilateral)			
	Right thalamus			
Brody *et al*. (2004) (*39*)	Right prefrontal cortex	24	42	32
	Right prefrontal cortex	16	50	32
	Right prefrontal cortex	8	62	28
	Right prefrontal cortex	34	6	48
	Right prefrontal cortex	20	34	52
	Right prefrontal cortex	32	18	54
	Right prefrontal cortex	32	18	54
	Left prefrontal cortex	–32	40	36
	Left prefrontal cortex	–52	30	36
	Cerebellum	16	–56	–26
Gallinat *et al*. (2006) (*40*)	Cingulate gyrus	6	4	32
	Thalamus	16	–24	–4
	Angular gyrus	43	–68	30
	Medial frontal gyrus	0	–9	47
	Cuneus	12	–73	23
	Inferior frontal gyrus	–31	16	–18
	Fusiform gyrus	–52	–21	–21
	Medial frontal gyrus	–9	40	26
	Parahippocampal gyrus	–12	–33	–3
	Superior temporal gyrus	–54	–24	3
	Inferior frontal gyrus	32	15	–20
	Cuneus	–9	–82	20
	Postcentral gyrus	–46	–31	52
	Lingual gyrus	14	–71	5
	Medial frontal gyrus	–2	19	–17
	Lingual gyrus	–14	–84	0
	Posterior cingulate	–8	–52	5
Zhang *et al*. (2010) (*41*)	Left prefrontal cortex	–24	–23	21
Zhang *et al*. (2011) (*42*)	Left insula	–38	–14	–7
	Left prefrontal cortex	–24	–46	32

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