

Effect of long-term cannabis use on axonal fibre connectivity

Andrew Zalesky,¹ Nadia Solowij,² Murat Yücel,¹ Dan I. Lubman,³ Michael Takagi,¹ Ian H. Harding,¹ Valentina Lorenzetti,¹ Ruopeng Wang,⁴ Karissa Searle,¹ Christos Pantelis¹ and Marc Seal⁵

1 Melbourne Neuropsychiatry Centre, The University of Melbourne and Melbourne Health, Melbourne, 3053, Australia

2 School of Psychology, University of Wollongong, Wollongong, 2522, Australia

3 Turning Point Alcohol and Drug Centre, Eastern Health and Monash University, Melbourne, 3065, Australia

4 Martinos Center for Biomedical Imaging, Massachusetts General Hospital, Boston, MA 02129, USA

5 Murdoch Childrens Research Institute, Royal Children's Hospital Melbourne, 3052, Australia

Correspondence to: Associate Prof. Nadia Solowij,
School of Psychology,
University of Wollongong,
Northfields Avenue,
Wollongong,
NSW 2522, Australia
E-mail: nadia@uow.edu.au

Cannabis use typically begins during adolescence and early adulthood, a period when cannabinoid receptors are still abundant in white matter pathways across the brain. However, few studies to date have explored the impact of regular cannabis use on white matter structure, with no previous studies examining its impact on axonal connectivity. The aim of this study was to examine axonal fibre pathways across the brain for evidence of microstructural alterations associated with long-term cannabis use and to test whether age of regular cannabis use is associated with severity of any microstructural change. To this end, diffusion-weighted magnetic resonance imaging and brain connectivity mapping techniques were performed in 59 cannabis users with longstanding histories of heavy use and 33 matched controls. Axonal connectivity was found to be impaired in the right fimbria of the hippocampus (fornix), splenium of the corpus callosum and commissural fibres. Radial and axial diffusivity in these pathways were associated with the age at which regular cannabis use commenced. Our findings indicate long-term cannabis use is hazardous to the white matter of the developing brain. Delaying the age at which regular use begins may minimize the severity of microstructural impairment.

Keywords: cannabis; axonal connectivity; white matter; diffusion-weighted imaging; diffusion-tensor imaging

Introduction

The enduring neuropathological effects of long-term cannabis use are equivocal (Lorenzetti *et al.*, 2010; Martin-Santos *et al.*, 2010). Changes in tissue volume, morphology and composition have been reported in neuronal structures rich in cannabinoid receptors,

most notably the hippocampus (Matochik *et al.*, 2005; Medina *et al.*, 2007; Yücel *et al.*, 2008) and the cerebellum (Solowij *et al.*, 2011b; Cohen *et al.*, 2012). On the other hand, many studies have found no neuropathological effects (Block *et al.*, 2000; Tzilos *et al.*, 2005; Jager *et al.*, 2007).

The discovery of cannabinoid receptors in oligodendroglial cells (Molina-Holgado *et al.*, 2002) motivates a new line of investigation centred on white matter. In particular, cannabinoid receptors have been detected in white matter structures of the foetal and post-natal rat brain, including the corpus callosum, anterior commissure, fornix, stria terminalis and stria medullaris (Romero *et al.*, 1997). In adulthood, the density of cannabinoid receptors in these fibre-enriched structures has been found to diminish, eventually supplanted by the classical distribution of cannabinoid receptors (Romero *et al.*, 1997; Glass *et al.*, 1998), which includes the cerebellum, hippocampus and caudate-putamen. This points to a transient developmental period during which white matter structures might be particularly sensitive to exogenous cannabis exposure (Fig. 1). More generally, developing white matter may be at greater risk of damage due to its higher concentration of cannabinoid receptors relative to the mature brain, an important consideration given adolescence and young adulthood is the peak time of cannabis initiation. Down regulation of the endogenous cannabinoid system due to long-term cannabis exposure during this developmental period (Dalton *et al.*, 2010) may result in apoptosis of oligodendrocyte progenitors (Barres *et al.*, 1992; Molina-Holgado *et al.*, 2002) and thereby alter white matter development (Kumra, 2007; Solowij *et al.*, 2011b).

The aim of this study was to examine axonal fibre pathways in the human brain for evidence of microstructural alteration associated with long-term heavy cannabis use. It was hypothesized that the age when cannabis use became heavy and regular would be a factor determining the severity of any microstructural alteration.

To test this hypothesis, diffusion-weighted imaging, a MRI modality capable of elucidating axonal directionality and microstructure *in vivo*, was performed in 59 cannabis users with a history of longstanding, heavy use and 33 non-users. The diffusion-weighted imaging data were processed using a validated fibre tracking algorithm to create high-resolution white matter connectivity maps indicating the extent of axonal connectivity between thousands of distinct voxel pairs. A novel network-based statistic (Zalesky *et al.*, 2010a) was then applied to isolate voxel

pairs showing between-group connectivity differences. Measures of axonal microstructure were taken in axonal fibre pathways identified as aberrant and correlated with the age of regular cannabis use.

Few previous studies have used diffusion-weighted imaging techniques to investigate the enduring effects of cannabis use on white matter. Microstructural alteration in the corpus callosum (Arnone *et al.*, 2008), the arcuate fasciculus (Ashtari *et al.*, 2009) and white matter surrounding the hippocampus (Yücel *et al.*, 2010) have been reported previously, while two studies found no effect on white matter (Gruber *et al.*, 2005; Delisi *et al.*, 2006). Two other studies have reported widespread white matter alterations in binge drinking cannabis users (Jacobus *et al.*, 2009; Bava *et al.*, 2010), while a recent graph-based study found reduced brain network efficiency and increased clustering in adult cannabis users (Kim *et al.*, 2012). This study confers several key advantages over these prior studies. With 59 cannabis users, our sample size is substantially larger than the samples recruited in previous studies and is not confounded by concurrent alcohol abuse. Furthermore, unlike most of the previous studies that confined their investigations to predefined regions of interest, our data-driven strategy was not biased by a subjective choice of region of interest. These novel factors enabled us to precisely localize any axonal fibre pathways demonstrating microstructural alteration associated with long-term cannabis use.

Materials and methods

Participants

Cannabis users with a long history of heavy use ($n = 59$) and healthy non-using volunteers ($n = 33$) were recruited from the general community via a variety of advertisements. The minimum cannabis use criterion for entry to the study was use of cannabis at least twice a month for a minimum of 3 years; the sample recruited had substantially greater use than this, as evident in Table 1. Lifetime (historical and recent) cannabis use was assessed with a detailed structured interview that included a Timeline Follow Back procedure (Sobell and Sobell, 1992). The majority of users tested positive for cannabinoid metabolites in their urine, but this was not a criterion for entry to the study. Participants were asked to abstain from substance use at least 12 h before the neuroimaging session, which involved functional neuroimaging paradigms as well as the diffusion MRI reported here. The cannabis users reported a median 15 h abstinence from cannabis use. Groups were matched in terms of mean age, IQ (Wechsler Abbreviated Scale of Intelligence), years of education and gender (Table 1). Users had greater trait anxiety (State-Trait Anxiety Inventory) and depressive symptoms (Beck Depression Inventory) and lower Global Assessment of Functioning scores. The Global Assessment of Functioning is a 0- to 100-point numerical scale that constitutes Axis V of the Diagnostic and Statistical Manual of Mental Disorders-IV and was used to rate the social, occupational and psychological functioning of each participant. Lower Global Assessment of Functioning scores are typical of the general population of cannabis users and are most likely due to cannabis use impacting on functioning in daily life. The Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders-IV Axis I disorders was administered to users (patient edition) and non-users (non-patient edition),

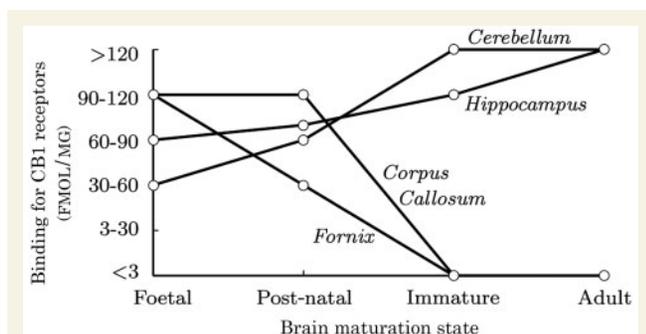


Figure 1 Unlike neuronal tissue, fibre-enriched structures become depleted of cannabinoid (CB) receptors as the rat brain matures. This leads to the hypothesis that axonal microstructure in the developing brain might be particularly reactive to exogenous cannabis exposure. Data extracted from Romero *et al.* (1997).

Table 1 Sample characteristics

Measure	Cannabis users (n = 59)	Non-users (n = 33)	Statistic (t)	P-value
Age (years)	33.4 (10.9)	31.5 (12.0)	0.8	0.5
IQ	109 (13)	111 (13)	0.6	0.6
Education (years)	13.1 (1.8)	13.1 (1.4)	1.6	0.1
Gender (males, %)	47	42	–	0.5
GAF	74 (10)	87 (4)	7.1	<0.001
STAI (anxiety)				
State	33 (9)	31 (10)	0.9	0.4
Trait	40 (13)	33 (8)	2.7	0.007
BDI (depression)	12 (10)	3 (3)	4.8	<0.001
SDS	5.5 (4.0)	–	–	–
Brain volume (cc)	1308.4 (114)	1304.9 (109)	0.1	0.9
Cannabis use				
Age started regular use (years)	16.7 (3.3)	–	–	–
Duration of regular use (years)	15.6 (9.5)	–	–	–
Current use (days/month)	25.7 (8.1)	–	–	–
Current use (joints/month)	147 (142)	–	–	–
Cumulative life dose (joints)	25 922 (25 838)	8 (16)	–	–
Cumulative dose past year (joints)	1880 (1382)	1 (3)	–	–
Alcohol use (standard drink/week)	5.5 (6.7)	4.1 (5.7)	1.0	0.3
Tobacco use (cigarettes/day)	9.4 (7.7)	1.0 (2.8)	6.1	<0.001

Values are mean (SD); all *P*-values are two-tailed. IQ estimated using the Wechsler Abbreviated Scale of Intelligence. Regular use defined as twice monthly or more. BDI = Beck Depression Inventory; GAF = Global Assessment of Functioning; SDS = Severity of Dependence Scale; STAI = State-Trait Anxiety Inventory.

to exclude those with a current or lifetime history of diagnosable medical, neurological or psychiatric conditions; other than cannabis dependence or abuse in the cannabis users. The other exclusion criterion was head injuries with concussion or without concussion but requiring hospitalization or treatment. Over the counter, non-opiate pain relief was allowed. Cannabis was the primary drug of use for all users, with limited experimental use of other illicit drugs, ascertained with a structured interview and urinalysis. The median number of cumulative lifetime episodes of other illicit drug use ranged from 0 to 4 for each of amphetamines, benzodiazepines, cocaine, ecstasy, hallucinogens, inhalants and opiates. Severity of Dependence Scale scores (Swift *et al.*, 1998) suggested at least moderate dependence in the majority of the sample (median 5, range 0–15). Users smoked a significantly greater amount of tobacco than non-users but did not differ in levels of alcohol consumption. Whole-brain volume did not differ between groups. Apart from one left-handed user, all users and non-users were right-handed. The study was approved by the Melbourne Health Research Ethics Committee and participant consent was obtained according to the Declaration of Helsinki.

Diffusion magnetic resonance imaging

Diffusion MRI (Le Bihan *et al.*, 2001) was used to quantify the diffusion of water molecules along multiple directions to infer microscopic details about white matter tissue architecture. A series of diffusion-weighted magnetic resonance images of brain anatomy were acquired in each participant using a Siemens Trio Magnetom 3T system located at the Murdoch Childrens Research Institute, Melbourne, Australia. Forty-two diffusion-weighted volumes were acquired using a spin-echo echo-planar imaging sequence with the following parameters: *b*-value, 2000 s/mm²; 54 consecutive axial slices of thickness 2.3 mm; 104 × 104 image matrix with an in-plane

voxel resolution of 2.3 × 2.3 mm; field of view, 24 × 24 cm; repetition time, 7400 ms; echo time, 106 ms; flip angle, 90°. Each volume provided a brain image quantifying the extent of water diffusion in a particular direction. Five non-diffusion-weighted volumes were also acquired.

Magnetic resonance imaging data preprocessing

Head movement and artefacts attributable to eddy currents were corrected using FMRIB Software Library software (<http://www.fmrib.ox.ac.uk/fsl/>). Gradient directions were not adjusted to account for any slight rotations performed to correct for head movement. The fractional anisotropy volume (Westin *et al.*, 2002) for each participant was computed and registered to 1 × 1 × 1 mm Montreal Neurological Institute (MNI) space using FLIRT (Jenkinson *et al.*, 2002) with 12 degrees of freedom. A two-phase registration process was used, whereby initial co-registration to the fractional anisotropy volume of a single representative participant (non-user) was followed by co-registration to the mean of the initially co-registered fractional anisotropy volumes. The rotation matrices resulting from the second phase were kept for later use in tractography registration. A binary brain mask indicating grey and white matter, but excluding CSF, was determined for each participant in native space by identifying upper and lower thresholds corresponding to minima in the histogram of the diffusion-weighted volumes.

Tractography

Millions of streamlines were generated to reconstruct the brain's entire network of axonal fibres. A streamline was a 3D curve that followed the trajectory of a particular fibre bundle. The interpolated streamline tracking method (Conturo *et al.*, 1999) with fixed increments of

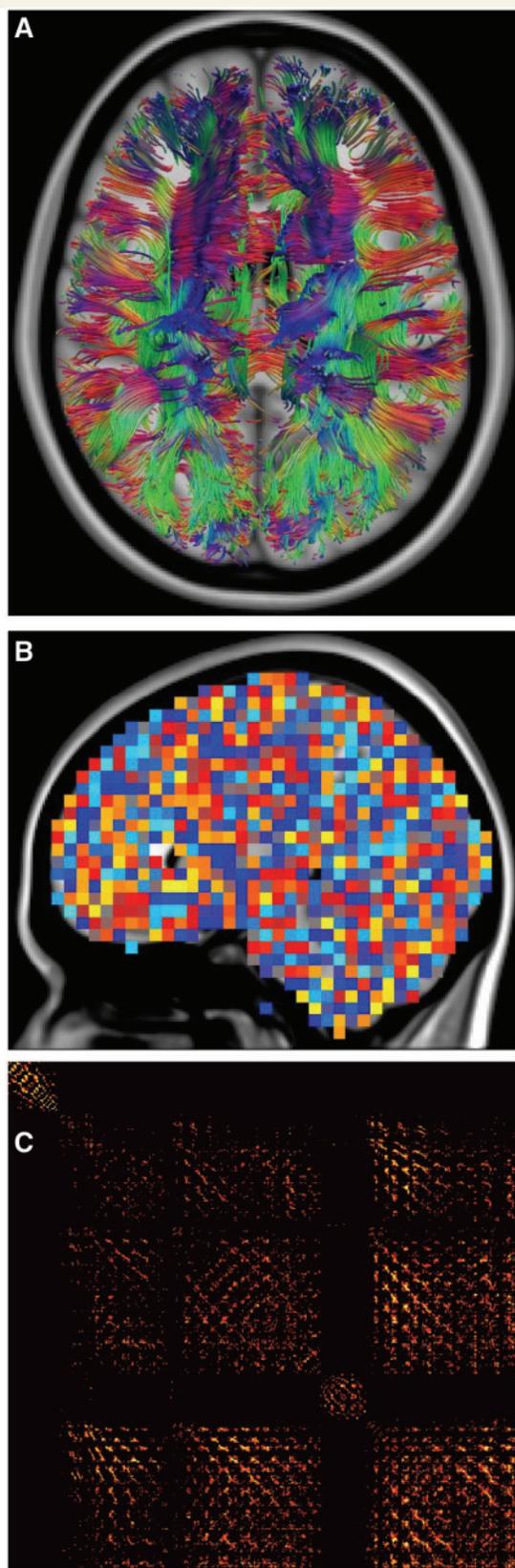


Figure 2 Overview of methodology. Whole-brain tractography was performed in each of 59 cannabis users and 33 non-users. (A) Tractography results were registered to standard space, shown here for a representative non-user. (B) Voxels of

1.15 mm was used to reconstruct axonal fibre trajectories in native space. Streamline propagation was guided by the direction of the interpolated principle eigenvector. Unless otherwise stated, the reconstructed trajectories followed the direction of maximum water diffusion (principle eigenvector), which is aligned with the path of any underlying axonal fibre bundles. Propagation was terminated at the first streamline increment either exceeding the participant's brain mask or violating a minimum angle threshold of 35° . A total of 12 streamlines were generated for each brain mask voxel, each of which was initiated from a random location in the voxel. Streamlines shorter than 10 mm were considered spurious and omitted. All remaining streamlines were co-registered to MNI space using the rotation matrices resulting from fractional anisotropy co-registration (Fig. 2A). Tractography was performed using Diffusion Toolkit (Wang *et al.*, 2007), while tractography registration was performed using in-house software.

Anatomical connectivity mapping

MNI space was subsampled using $5 \times 5 \times 5$ mm voxels, approximately double the native resolution (Fig. 2B). Each voxel was a small cube that encapsulated a distinct volume of brain tissue. A connectivity matrix (Bullmore and Sporns, 2009; He and Evans, 2010) was then mapped for each participant, where each row/column of the connectivity matrix indexed a unique $5 \times 5 \times 5$ mm voxel. The connectivity matrix was populated so that cell (i, j) enumerated the total number of streamlines with one endpoint in voxel i and the other in voxel j . This provided a measure of the extent to which voxel i and voxel j were anatomically connected (Zalesky *et al.*, 2011). In other words, each cell in the connectivity matrix stored a single value quantifying the extent of axonal connectivity between a pair of spatially distant brain regions. Streamlines with both endpoints in the same voxel were disregarded. The connectivity matrices were summed cell-wise over all participants. Rows/columns indexing voxels visited by fewer than 100 streamline endpoints in the summed connectivity matrix were deleted. Deleted voxels predominantly comprised CSF or resided outside brain matter. The end result was a $14\,398 \times 14\,398$ connectivity matrix for each participant quantifying the extent of anatomical connectivity between all possible pairs of grey and white matter voxels (Fig. 2C). Connectivity matrices were computed using in-house software.

This approach is an enhancement of template-driven methods (Hagmann *et al.*, 2008; Zalesky *et al.*, 2010b, 2011) for mapping anatomical connectivity. Rather than mapping connectivity between large grey matter regions defined by a parcellation template, connectivity was mapped between all grey and white matter voxels. This prevented bias owing to the use of an arbitrary template (Wig *et al.*, 2011). High resolution connectivity mapping has already been demonstrated with functional brain imaging techniques (van den Heuvel *et al.*, 2008; Hayasaka and Laurienti, 2010).

Furthermore, including white matter voxels enabled detection of streamlines that terminated before reaching grey matter, either due to axonal pathology or an inaccurately delineated grey–white

dimension 5 mm isotropic covered the entire cortex, twice greater than native resolution. The total number of streamlines interconnecting each voxel pair was enumerated, yielding a $14\,398 \times 14\,398$ connectivity matrix (C, only portion shown). Voxel pairs for which the streamline count differed significantly between cannabis users and non-users were identified with the network-based statistic.

boundary. With template-driven methods, a streamline terminating just before the grey–white boundary is disregarded because its endpoint does not reside in a grey matter region. Disregarding a streamline that terminates just before the grey–white boundary can be considered undesirable because the precise point at which a streamline terminates is determined by arbitrary criteria, the grey–white boundary can be difficult to accurately delineate and premature termination can serve as an indicator of axonal pathology. For example, premature termination due to axonal pathology may be indicated by an increased streamline count at the white matter voxel containing the point of premature termination and/or a decreased count at the downstream endpoint that would have been reached in the absence of pathology.

Subsampling to voxels of size $5 \times 5 \times 5$ mm was performed to ensure a sufficiently dynamic range of streamline counts. At higher resolutions, a participant's connectivity matrix was predominantly populated with values of 0 and 1, but rarely with values exceeding 1. This was considered too narrow a dynamic range to enable statistical analysis and may have resulted in greater type II error.

Statistical analysis

Statistical analysis was performed to identify voxel pairs between which the extent of anatomical connectivity was significantly greater/lower in users compared to non-users. The null hypothesis tested was equality in the average streamline count between both groups. The *t*-statistic was used to test the null hypothesis for each of the 169 863 voxel pairs that were interconnected by at least one streamline on average. Other voxel pairs were not tested to ease the computational burden and reduce the number of multiple comparisons. This was not to the detriment of type II error because rejection of the null hypothesis was unlikely for a voxel pair interconnected by less than one streamline on average, in which case the streamline count rarely differed from the values of one and zero. The network-based statistic was used to control the family-wise error rate among the 169 863 hypotheses tested. Controlling the family-wise error rate ensured the probability of making one or more false discoveries (type I errors) among all the hypotheses was less than the nominal error rate of $\alpha = 0.05$ (Nichols and Hayasaka, 2003).

The network-based statistic (Zalesky *et al.*, 2010a, 2012) is a non-parametric multiple comparisons procedure for identifying network connectivity differences. It has been used to map functional (Fornito *et al.*, 2011; Zhang *et al.*, 2011) and axonal (Verstraete, 2011; Zalesky *et al.*, 2011; Bai *et al.*, 2012) connectivity disturbances in psychiatric and neurological disease. The network-based statistic was used in accordance with these prior studies. In particular, voxel pairs, referred to as 'links', with a *t*-statistic exceeding an uncorrected threshold of 3 ($P = 0.001$) were first admitted into a set of supra-threshold links. Connected components that were present in the set of supra-threshold links were then identified using a breadth first search (Ahuja *et al.*, 1993) and the number of links each component comprised was stored (Zalesky *et al.*, 2010a). A 'connected component' in this context was a set of voxels in which any two voxels were connected to each other via a path comprising supra-threshold links. Two distinct connected components were found (refer to 'Results' section), each comprising five links (i.e. five supra-threshold voxel pairs).

A family-wise error corrected *P*-value was ascribed to each component using permutation testing on the number of links it comprised. This *P*-value quantified the probability of finding a connected component comprising five or more links under the null hypothesis of no between-group difference. Permutation testing was performed by

randomly exchanging users and non-users, such that some users were labelled non-users and vice versa. A set of supra-threshold links was determined for the randomized data using the same *t*-statistic threshold of 3 and the number of links comprising the largest connected component was stored. Four thousand permutations were generated to yield an empirical null distribution for the size of the largest connected component. A corrected *P*-value was then calculated as the proportion of permutations for which the largest connected component comprised five or more links. Permutations were generated randomly and no permutation was excluded.

It is important to note that statistical inference with the network-based statistic is performed at the resolution of connected components. In other words, the null hypothesis can be rejected for each connected component as a whole, but not for individual voxel pairs (i.e. links) comprising a component. This is sometimes referred to as control of the family-wise error rate in the weak sense (Nichols and Hayasaka, 2003).

Streamlines comprising the two significant networks ($P < 0.05$, corrected) were isolated and visualized using TrackVis (Wang *et al.*, 2007). Two distinct indices of white matter microstructure, axonal and radial diffusivity (Wheeler-Kingshott and Cercignani, 2009), were averaged over all voxels intersected by at least one streamline. This yielded a network-wide measure of radial and axial diffusivity for each significant network. Radial and axial diffusivity were regressed against the age at which regular cannabis use commenced, cumulative life dose and duration of regular use, with biological age included as a nuisance regressor to control for maturation effects.

Results

Brain-wide differences

A total of 1.19 ± 0.1 million streamlines were generated for each participant. No between-group difference was found in the total number of streamlines (users: 1.19 ± 0.10 million, non-users: 1.19 ± 0.11 million; $t = 0.1$, $P = 0.9$), indicating no evidence of brain-wide connectivity disturbances. Streamlines were stratified according to length into 10 mm bins, with the first bin containing lengths between 0 and 10 mm. No between-group differences were found in the number of streamlines comprising any of the 20 bins, indicating no evidence of any brain-wide shortening of axonal fibres. No between-group difference was found in whole-brain volume (Table 1).

Localized differences

The network-based statistic identified two localized networks (i.e. connected components) interconnected with significantly fewer streamlines in users compared to non-users, indicating tract-specific disturbances in axonal integrity. The first network (Fig. 3) implicated the right fimbria (crus of the fornix), a prominent band of fibres running alongside the medial periphery of the hippocampus, as well as the splenium of the corpus callosum bilaterally. The fimbria can be seen to extend medially towards the hippocampal commissure, but did not continue into the left hemisphere. The fimbria network comprised a total of 7 ± 1.6 streamlines in users (mean \pm standard error) and 44 ± 6.4 in non-users, an 84% reduction. The second network (Fig. 4) exclusively

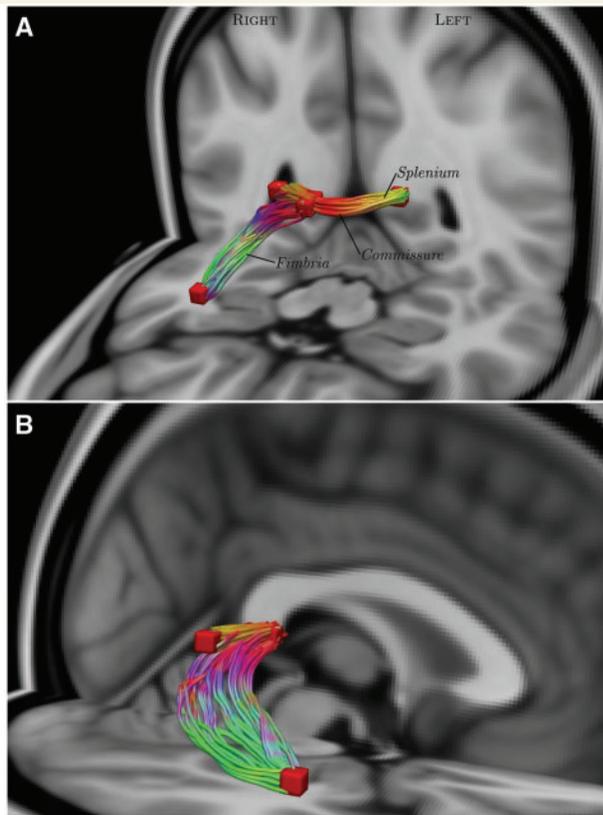


Figure 3 The right fimbria of the hippocampus, hippocampal commissure and splenium comprised fewer streamlines in cannabis users compared to non-users ($P < 0.05$, corrected). Voxels interconnected by fewer streamlines are coloured red and the corresponding streamlines via which they are interconnected are coloured such that: left–right is red, superior–inferior is blue and anterior–posterior is green. **A** and **B** show different oblique views. The splenium and hippocampal commissure are obscured in **B** by the sagittal slice of the underlay image.

implicated a commissural fibre beginning at the splenium of the corpus callosum and extending deep within the right precuneus. The commissural fibre comprised a total of 9 ± 1.7 streamlines in users and 78 ± 15.1 in non-users, an 88% reduction. Both networks comprised five voxel pairs, each of which has been depicted as a pair of solid red cubes. The probability of finding a network comprising five voxel pairs as a matter of chance satisfied $P < 0.05$, established with permutation testing. No significant gender differences or gender by group interactions were found, although the reduction in streamline count between male users and non-users was greater than the reduction between female users and non-users at trend level significance ($P = 0.07$, permutation testing) in the commissural fibre.

No network was found to be interconnected with significantly greater streamlines in users compared to non-users.

Post hoc analysis

Regression was used to assess the confounding effects of alcohol, tobacco, trait anxiety and depressive symptoms. Separate

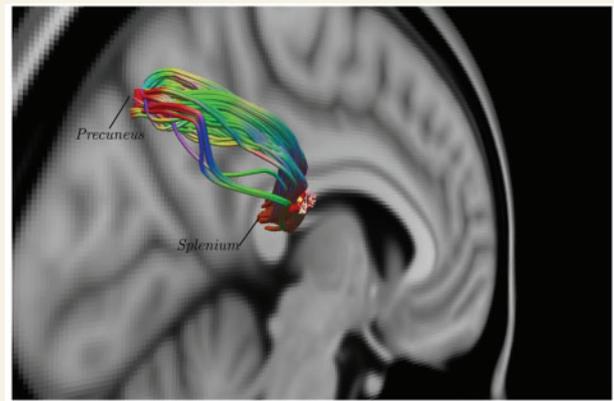


Figure 4 Fewer streamlines interconnected the right precuneus with the splenium in cannabis users compared to non-users ($P < 0.05$, corrected). Voxels interconnected by fewer streamlines are coloured red and the corresponding streamlines via which they are interconnected are coloured such that: left–right is red, superior–inferior is blue and anterior–posterior is green.

regression analyses were performed for the two networks interconnected with significantly fewer streamlines. The dependent variable was the streamline count. Alcohol use (standard drinks/week), tobacco use (cigarettes/day), trait anxiety (State-Trait Anxiety Inventory) and depressive symptoms (Beck Depression Inventory) were included as nuisance regressors. Group membership (user/non-user) was the regressor of interest and was the only significant regressor for both networks (fimbria: $P < 0.001$; commissural: $P < 0.001$). Alcohol (fimbria: $P = 0.9$; commissural: $P = 0.8$), tobacco (fimbria: $P = 0.7$; commissural: $P = 0.8$), trait anxiety (fimbria: $P = 0.1$; commissural: $P = 0.8$) and depressive symptoms (fimbria: $P = 0.8$; commissural: $P = 0.9$) were not significant regressors.

Correlation analysis

Radial and axial diffusivity were positively correlated with the age at which regular cannabis use commenced in both the fimbria and commissural fibre (Fig. 5) indicating that the enduring effect of cannabis use on axonal microstructure was mediated by this factor. The associations were highly significant in the commissural fibre (radial and axial: $P < 0.001$) and marginally significant in the fimbria (radial: $P = 0.02$, axial: $P = 0.06$). The associations remained significant after controlling for biological age as well as the duration of regular cannabis use. No associations with duration of regular cannabis use and cumulative life dose were found. No association was found between the duration of regular cannabis use and the age at which regular cannabis use commenced.

Discussion

This is the first study to comprehensively investigate axonal fibre connectivity among individuals with a history of longstanding, heavy cannabis use. Axonal connectivity was found to be impaired

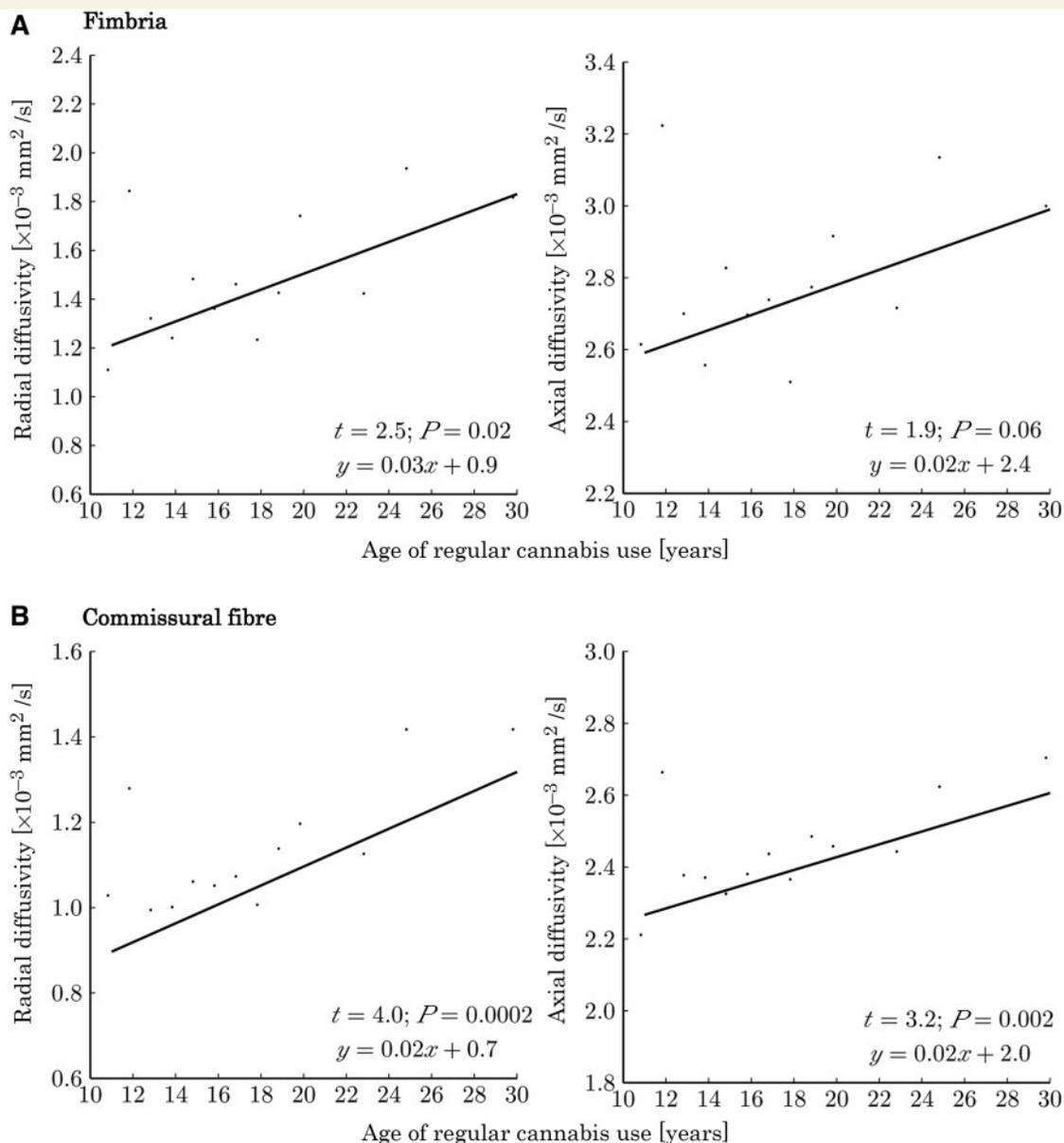


Figure 5 Age at which regular cannabis use commenced was correlated with radial and axial diffusivity in the fimbria (A) and commissural pathways extending to the precuneus (B). Biological age was included as a nuisance covariate. Each data point, depicted as a cross, represents the average of the diffusivity measure over all cannabis users of the same age of regular use.

in the right fimbria of the hippocampus (fornix), splenium of the corpus callosum and commissural fibres extending to the precuneus. The fornix and corpus callosum are two of only a very small number of fibre-enriched structures that have abundant cannabinoid receptors in the developing rat brain (Romero *et al.*, 1997; Molina-Holgado *et al.*, 2002). All users in this study commenced regular use during adolescence or early adulthood, a time of continuing white matter development (Barnea-Gorlay *et al.*, 2005). As such, our findings are particularly compelling given that our whole-brain investigation of white matter exclusively localized some of the very few fibre pathways that have abundant cannabinoid receptors during brain development.

This is also the first study to demonstrate that the age at which regular cannabis use begins is a key factor determining the severity of any microstructural white matter alteration. Radial and axial diffusivity were both positively associated with the age at which regular use commenced. Caution should be exercised when interpreting this result in terms of underlying tissue microstructure. An increase in radial diffusivity, which reflects the diffusion of water perpendicular to a fibre, is generally considered a marker of reduced myelination (Song *et al.*, 2005). In contrast, a decrease in axial diffusivity, which reflects the diffusion of water parallel to a fibre, is often considered a marker of axonal injury (Budde *et al.*, 2009). In some circumstances, however, a *bona fide* decrease in

axial diffusivity can result in a commensurate decrease in radial diffusivity (Wheeler-Kingshott and Cercignani, 2009).

This was the situation encountered in this study and was most likely the result of crossing fibres, eigenvalue sorting bias (Pierpaoli and Basser, 1996) or microstructural geometry that was not fully characterizable with the diffusion tensor model. As such, it was difficult to unequivocally ascertain a microstructural basis for the association with the age at which regular use commenced. This should not be misconstrued as evidence against the validity or statistical significance of the association itself. The association presents compelling evidence for white matter reacting differently to cannabis exposure commencing during adolescence compared with adulthood, most likely due to the high concentration of cannabinoid receptors contained within structures, such as the corpus callosum and fornix during adolescence. The association remained significant when biological age and cumulative lifetime exposure were included as nuisance regressors, arguing against simple brain maturation or dose-dependent effects. Using a crossing fibre model (Tournier *et al.*, 2008) may have enabled better characterization of the association's microstructural basis; however, the MRI parameters used in this study were not ideally suited to use of such models.

This result accords with an earlier study that reported an analogous association between fractional anisotropy and age of regular cannabis and inhalant use among adolescent users (Yücel *et al.*, 2010). It is also in line with a recent study reporting that the age of onset of cannabis use positively correlated with measures of frontal fractional anisotropy and inversely with mean diffusivity (Gruber *et al.*, 2011). Our finding also supports mounting evidence suggesting a link between adolescent cannabis use and schizophrenia in later life (Rais *et al.*, 2008; Peters *et al.*, 2009; Dekker *et al.*, 2010; Ho *et al.*, 2011; James *et al.*, 2011) as well as with evidence for greater adverse cognitive effects in adolescent cannabis users (Solowij *et al.*, 2011a, 2012). An earlier study (Arnone *et al.*, 2008) using a sophisticated region of interest approach also found evidence of microstructural alteration in the corpus callosum of cannabis users. More generally, a review of diffusion tensor imaging studies in alcohol, cannabis and cocaine addiction noted focal disruption of commissural connectivity in the corpus callosum (Arnone *et al.*, 2006). Previous studies investigating white matter among cannabis users have recruited substantially smaller samples. With a sample size of 59, this study is better positioned to resolve the lack of consistency in findings reflected across these prior studies. Furthermore, unlike most of the previous studies that confined their investigations to predefined regions of interest, the novel data-driven strategy used in this study was not biased by a subjective choice of regions of interest. All axonal fibre pathways were interrogated using a powerful exploratory approach controlling for the family-wise error rate.

The size of the connectivity differences in per cent terms was substantial, with an 84% reduction in streamline count in the fimbria and an 88% reduction in the commissural pathways extending to the precuneus. This should not be interpreted as evidence for a substantial reduction in the absolute number of axonal fibres comprising these structures, since a streamline is not tantamount to an individual axon. The precise microstructural underpinnings of an altered streamline count are not well understood, hence the

reason for using the more interpretable measures of radial and axial diffusivity in the correlational analysis. Nevertheless, the substantial differences suggest these results are robust and reproducible.

Several potential limitations must be noted. Tractography was used to create high-resolution white matter connectivity maps indicating the extent of axonal connectivity between thousands of distinct voxel pairs. The accuracy of these connectivity maps was contingent on the reliability of the fibre tracking method. Fibre trajectories were tracked by following the principle direction of water diffusion indicated by the diffusion tensor (Conturo *et al.*, 1999). While this is a reliable tracking method known to yield reproducible results (Catani *et al.*, 2002), some fibres may have been overlooked due to inaccuracy of the diffusion tensor model in regions of complex fibre geometry (e.g. crossing fibres), partial volume effects or distal/flare bias (Zalesky *et al.*, 2009). Between-group differences could not be tested at fibres that were not tracked, thus representing a minimal risk for type II error. Another potential risk for type II error was the narrow range of streamline counts evident for some voxel pairs. Streamline counts that rarely exceeded the values of 0 and 1 across individuals provide less sensitivity than a wider and dynamic range of counts. This range was widened by increasing the overall total number of streamlines generated, although beyond a certain increase, the stochastic diversity of the data was exhausted and computational tractability became a concern. The risk of committing a type II error due to this factor was minimal because very low streamline counts were most likely indicative of spurious tracking results.

While cannabis use was ascertained primarily by self-report (corroborated by urinalysis), self-report measures are generally reliable (Harrison, 1997; Buchan *et al.*, 2002) and have been shown to be associated with brain structural alterations in a range of studies (Matochik *et al.*, 2005; Yücel *et al.*, 2008; Cousijn *et al.*, 2012). Cannabis users had significantly greater trait anxiety and depressive symptoms, and smoked significantly greater amounts of tobacco than non-users. However, no participant had ever been diagnosed with an anxiety or depressive disorder or had sought treatment for such symptoms. While it is possible that reported between-group differences were mediated by differences in trait anxiety, depressive symptoms or tobacco use, a *post hoc* analysis revealed both connectivity differences remained significant after controlling for these variables.

The cannabis users of this study were asked to abstain from cannabis for at least 12 h (median self-reported abstinence: 15 h). Cannabinoid residues may affect cognition and measures of functional brain activation (Martin-Santos *et al.*, 2010; Solowij and Pesa, 2012). Diffusion tensor metrics, on the other hand, are thought to index structural/morphological integrity or abnormalities, not functional abnormalities. Further, these metrics tend to be stable over time (Vollmar *et al.*, 2010), and would be less affected by transient states, such as residual intoxication or withdrawal than are neurocognitive or functional brain activation measures. Structural or morphological abnormalities may, however, have functional consequences. It is possible that the white matter abnormalities associated with cannabis use could be reversible given a sufficient period of abstinence or functional

adaptation. An important avenue for future research is to clarify whether abstinence can lead to recovery of axonal injury and the associated time-course.

Our results suggest that long-term cannabis use is hazardous to white matter in the developing brain. Given the association between cannabis-related harms and age of onset of regular use, delaying use may minimize such harmful effects. Disturbed brain connectivity in cannabis users may underlie cognitive impairment and vulnerability to psychosis, depression and anxiety disorders (Lim *et al.*, 2002), all of which are significant public health concerns. White matter alterations have been associated with various functional and clinical outcomes in schizophrenia, including illness, symptomatic and cognitive measures (Walterfang *et al.*, 2011), with white matter pathology underlying faulty integration of cortical–cerebellar–thalamic–cortical circuits thought to play a primary role in the observed cognitive deficits (Wexler *et al.*, 2009). Similar connectivity disturbances, particularly in the fimbria of the hippocampus and commissural fibres extending to the precuneus reported in this study, may underlie the memory impairment and other cognitive deficits that are observed in long-term heavy cannabis users (Solowij *et al.*, 2011b; Solowij and Pesa, 2012). The effect of long-term cannabis use on functional brain connectivity (van den Heuvel and Hulshoff Pol, 2010) and network topology (He and Evans, 2010; Rubinov and Sporns, 2010) remains an important avenue to pursue.

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