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Alteration to hippocampal volume and shape confined to cannabis dependence: a multi-site study

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ABSTRACT

Cannabis use is highly prevalent and often considered to be relatively harmless. Nonetheless, a subset of regular cannabis users may develop dependence, experiencing poorer quality of life and greater mental health problems relative to non-dependent users. The neuroanatomy characterizing cannabis use versus dependence is poorly understood. We aimed to delineate the contributing role of cannabis use and dependence on morphology of the hippocampus, one of the most consistently altered brain regions in cannabis users, in a large multi-site dataset aggregated across four research sites. We compared hippocampal volume and vertex-level hippocampal shape differences (1) between 121 non-using controls and 140 cannabis users; (2) between 106 controls, 50 non-dependent users and 70 dependent users; and (3) between a subset of 41 controls, 41 non-dependent users and 41 dependent users, matched on sample characteristics and cannabis use pattern (onset age and dosage). Cannabis users did not differ from controls in hippocampal volume or shape. However, cannabis-dependent users had significantly smaller right and left hippocampi relative to controls and non-dependent users, irrespective of cannabis dosage. Shape analysis indicated localized deflations in the superior-medial body of the hippocampus. Our findings support neuroscientific theories postulating dependence-specific neuroadaptations in cannabis users. Future efforts should uncover the neurobiological risk and liabilities separating dependent and non-dependent use of cannabis.

Keywords brain, cannabis, dependence, hippocampus, MRI, neuroimaging, substance use.

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INTRODUCTION

Cannabis use has been widespread globally over the past two decades, with the most recent census estimating a prevalence of up to 183 million users (United Nations Office on Drugs and Crime 2016). This number may increase with recent legislative changes and more liberal policies surrounding both recreational and medicinal cannabis use, fueling debate on public health consequences, such as the potential increase in cannabis dependence and cannabis-related problems (Hasin *et al.* 2017). Despite a general community perception of harmlessness, a subset of regular cannabis users—over 13 million—are dependent on cannabis (Degenhardt *et al.* 2013; United Nations Office on Drugs and Crime 2016). In addition, almost 50 percent of substance users seeking treatment are cannabis dependent (United Nations Office on Drugs and Crime 2016). Cannabis dependence represents a significant burden on the individual and society but has been poorly defined

neurobiologically compared with heavy, non-dependent use. This warrants greater attention to distinctions between cannabis use and dependence and associated harms and vulnerabilities.

Individuals with cannabis dependence report diminished control over use and compulsive use despite associated negative consequences to their functioning and mental health (American Psychiatric Association 2013; van der Pol *et al.* 2013a). Relative to non-dependent users, they also experience greater mental health issues (i.e. mood, anxiety and conduct disorder) (van der Pol *et al.* 2013a) and impaired cognitive functioning in the domains of learning, working memory and cognitive flexibility (Solowij & Battisti 2008; Broyd *et al.* 2016). Such impaired functioning may be underpinned by neuroanatomical alterations across brain regions relevant to motivation, emotion and cognition (Koob 2009; Chambers 2013), as demonstrated in regular cannabis users with higher levels of use and problem use (Koenders *et al.* 2016; Lorenzetti, Solowij, & Yücel 2016c). In particular, the hippocampus is often suggested to be affected by cannabis users, with a number of studies reporting hippocampal volume to be reduced in regular cannabis users relative to non-users (Yücel *et al.* 2008; Ashtari *et al.* 2011; Demirakca *et al.* 2011; Rocchetti *et al.* 2013; Koenders *et al.* 2016; Yücel *et al.* 2016). However, almost as many studies have not observed cannabis-use-related hippocampal alterations (Tzilos *et al.* 2005; Medina *et al.* 2007; Gilman *et al.* 2014; Mashhoon *et al.* 2015; Weiland *et al.* 2015). The wide-ranging sample characteristics across studies (e.g. average duration of use range from 3 to 20 years; average age of user sample range from 20 to 40 years old), the small sample size of individual studies (i.e. range of cannabis-using sample from 11 to 61) and the lack of consideration of cannabis dependence preclude identification of key factors involved in specific hippocampal aberrations.

Emerging evidence demonstrates differences between non-dependent and dependent cannabis users in brain structure [i.e. orbitofrontal cortex and hippocampal volume (Chye *et al.* 2017a; Chye *et al.* 2017b)] and brain function [i.e. functional connectivity across amygdala, anterior cingulate, orbitofrontal cortex, hippocampus and nucleus accumbens (Filbey & Dunlop 2014)]. Such findings may reflect neural adaptations that discriminate compulsive use in substance dependence (Koob 2009; Chambers 2013; Koob & Volkow 2017). However, most previous studies of regular cannabis users have not clarified the differences specific to dependence versus non-dependence in regular cannabis users. It is important to distinguish between these groups to improve identification and prevention in user populations most vulnerable to cannabis-related harms.

We aimed to delineate the contributing roles of cannabis use and dependence on the hippocampus, one of the most consistently reported brain regions to be altered in cannabis users (Lorenzetti *et al.* 2016c), by re-examining hippocampal morphology across an aggregated sample of 261 cannabis users (dependent and non-dependent) and non-using controls (CON) from four research sites globally (Batalla *et al.* 2013; Solowij *et al.* 2013; Cousijn *et al.* 2014; Yücel *et al.* 2016). While the aforementioned study findings have been mixed in relation to hippocampal morphology in diverse cannabis using samples, none of these studies specifically examined cannabis dependence relative to non-dependent heavy use. We compared hippocampal morphology (i.e. both volume and shape) between (1) regular cannabis users (CB) and non-using CON and between (2) dependent users (CB-dep), non-dependent users (CB-nondep) and CON. To validate potential dependence-related hippocampal morphological differences, we further examined hippocampal volume and shape between (3) a subset of CB-dep, CB-nondep and CON, matched on age, gender distribution, intelligence quotient (IQ) and alcohol use, with CB-dep and CB-nondep further matched on tobacco use and cannabis use (i.e. onset and dosage). We hypothesized that hippocampal volume reduction and shape alteration would be apparent in regular cannabis users (both CB-dep and CB-nondep) relative to CON and that these effects would be more pronounced in CB-dep relative to CB-nondep.

METHOD

Participants

Participants comprising 121 CON (aged 18 to 55; *Mdn* = 24 years) and 140 CB (aged 18 to 56; *Mdn* = 24 years) were recruited from four independently conducted studies across Amsterdam (N = 76; Cousijn *et al.* 2014), Barcelona (N = 55; Batalla *et al.* 2013), Wollongong (N = 30; Solowij *et al.* 2013) and Melbourne (N = 100; Yücel *et al.* 2016). Inclusion and exclusion criteria have been documented in a previous paper (Chye *et al.* 2017a) and in the Supplementary Table S1. Briefly, CB had to have used cannabis at least 2 days/month for at least 2 months, although most CB had almost daily cannabis use for a considerable period of time (duration of regular use, *Mdn* = 6 years, *range* = 0.5–38 years; lifetime use, *Mdn* = 15 690 cones, *range* = 600–864 000 cones). Meanwhile, CON used less than 50 times in their lifetime and did not use in the past month. All subjects had no history of chronic medical illness or neurological conditions or any lifetime Axis I psychiatric disorder

apart from nicotine use disorder or cannabis use disorder and had minimal illicit substance use other than cannabis (<50 times in the past 10 years).

Measures

Participants' demographic and substance use characteristics were assessed separately at each individual research site. Select information [i.e. age, gender, IQ, monthly tobacco (cigarettes) use, monthly standard alcoholic drinks and cannabis use measures] was subsequently standardized across sites (refer measures in Supplementary Table S1). Cannabis use measures included monthly and lifetime cannabis consumption (measured in cones, https://cannabissupport.com.au/media/1593/timeline-followback.pdf), age of initiation of regular cannabis use and cannabis dependence.

Cannabis dependence information was only available from three of the four sites and was used to separate the aggregated three-site sample into 70 CB-dep, 50 CB-nondep and 106 CON based on recommended norms and after excluding subjects with missing dependence information. Specifically, in Amsterdam, the Mini International Neuropsychiatric Interview's 'non-alcohol psychoactive substance use disorders' module was used, with a cut-off of 3 and above as CB-dep (Lecrubier *et al.* 1997), while Barcelona and Melbourne used the Severity of Dependence Scale, with a cut-off of 4 and above as CB-dep (Gossop *et al.* 1995).

Structural image processing

T1-weighted structural magnetic resonance images were acquired separately from each research site. Scanner details have been documented previously (Batalla *et al.* 2013; Solowij *et al.* 2013; Cousijn *et al.* 2014; Yücel *et al.* 2016; Chye *et al.* 2017a), as well as in Supplementary Table S1. Two sites used a Phillips Intera 3T scanner with an 8-channel head coil (Amsterdam and Wollongong), one site used a GE Signa Excite 1.5T scanner with an 8-channel head coil (Barcelona) and one site used a Siemens-Trio 3T scanner with a 32-channel head coil (Melbourne).

Magnetic resonance images were corrected for intensity inhomogeneity—nonparametric nonuniform intensity normalization (Sled, Zijdenbos, & Evans 1998) using FreeSurfer image analysis (http://surfer.nmr.mgh.harvard.edu/) version 5.3.0. An estimate of the intracranial volume (ICV) was also obtained from FreeSurfer's automated parcellation procedure. Subsequently, the images' intensity was standardized across sites, based on the average gray matter, white matter and cerebrospinal fluid intensity from each site, using the FMRIB Software Library (FSL; http://www.fmrib.ox.ac.uk/fsl/). Finally, the images were visually inspected to ensure consistent orientation along the anterior commissure–posterior commissure plane.

Volumetric analysis

The hippocampus was manually traced by a trained tracer (Y. C.) blinded to group and site membership, using the Analyze 12.0 software (AnalyzeDirect, Overland Park, KS), according to a validated protocol (Velakoulis *et al.* 1999). Hippocampal boundaries were defined posteriorly as the slice with the greatest length of continuous fornix; medially as the open end of the hippocampal fissure (posterior) and the uncal fissure (anterior); laterally as the temporal horn of the lateral ventricle; inferiorly as the parahippocampal white matter; and superiorly as the fimbria and alveus (posterior) as well as the amygdala (anterior).

Intra-rater and inter-rater reliabilities (i.e. intraclass correlation coefficient, absolute agreement and single measures) for the hippocampal tracing were assessed on 28 randomly selected images. Intra-rater reliabilities for the right and left hippocampus were 0.97 and 0.88, respectively, while inter-rater reliabilities against an expert tracer (V. L.) were 0.90 and 0.93, respectively. Intra-rater reliability was also consistent across scanner field strength, at an intraclass correlation coefficient of 0.95 (collapsed across both hemispheres) for both 1.5T and 3T scanners. As tracing of all 261 images proceeded over a 4-month period (from April to August 2016), longitudinal intra-rater reliability was performed on 15 images (i.e. five images repeated 3 times, evenly distributed across the blinded sample). Values were 0.93 and 0.83 for the right and left hippocampus, respectively, indicating good consistency over time.

A series of univariate analysis of covariance models were run to examine the association between cannabis use and dependence and left and right hippocampus volume. This included (1) comparison between CON and CB, controlling for imaging site as random factor, gender as fixed factor, and ICV, age, IQ and monthly alcohol and tobacco use as covariates; (2a) comparison between CON, CB-nondep and CB-dep (only from the three sites that obtained dependence measures—Amsterdam, Barcelona and Melbourne), controlling for all previously mentioned variables; (2b) comparison between CB-nondep and CB-dep users only, with additional inclusion of all cannabis use measures (current monthly cones, lifetime cones and age of regular use) as covariates; and (3) comparison between CON, CB-nondep and CB-dep, in a subset of subjects matched on gender, age, IQ and alcohol use across all groups and matched on tobacco and cannabis use (current monthly cones,

lifetime cones and age of regular use) across CB-nondep and CB-dep.

Shape analysis

The manually traced hippocampal boundaries (i.e. object maps) were used to run shape analysis within FSL. First, the object maps were registered to Montreal Neurological Institute space, with reference from their respective T1-weighted images. Next, average boundary images for the hippocampal object maps (separately for the right and left hippocampus) were obtained. To do this, we first averaged the object maps together and binarized them at the 60 percent threshold. From this, we formed a 1-voxel-thick average boundary shape by subtracting away an eroded version of the threshold-shape. The signed distance of each individual hippocampal object to every point on the average boundary shape could then be calculated. A flow chart of the shape analysis processing steps is presented in Supplementary Fig. S1.

The signed distances for each hippocampal label were used for further statistical analysis. A permutation-based approach with threshold-free cluster enhancement was adopted using FSL's randomize tool (Smith & Nichols 2009; Winkler *et al.* 2014). A total of 100 000 permutations were used for the analysis, examining shape differences between (1) CON versus CB and (2) CON versus CB-nondep versus CB-dep and (3) the matched subset of CON versus CB-nondep versus CB-dep, all controlling for imaging site, gender, age, IQ, alcohol use and tobacco use.

Automated segmentation versus manual tracing

Given that it is often unfeasible for all studies, particularly studies with large databases, to quantify brain structures via manual tracing, we further compared the performance of the automated tool—FreeSurfer in hippocampal segmentation, by replicating all volume and shape analysis. Hippocampal segmentation was performed by FreeSurfer version 5.3, as described by Fischl *et al.* (2002). Shape analysis was also performed with a similar processing step as presented in Supplementary Fig. S1. The automated segmentation procedure was also validated against our manual tracing, which is considered the gold standard for evaluating hippocampal volume (Velakoulis *et al.* 1999), by examining the (1) correlation between both methods and the (2) percent volume overlap (i.e. Dice coefficient, DICE) as defined by the equation

$$\mathrm{DICE} = \frac{\mathrm{V}(\mathrm{manual} \cap \mathrm{Freesurfer})}{(\mathrm{V}(\mathrm{manual}) + \mathrm{V}(\mathrm{freesurfer}))/2} \times 100.$$

RESULTS

Sample characteristics

Participant characteristics and hippocampal volume measures (1) by cannabis use (i.e. CON versus CB) and (2) by cannabis dependence (i.e. CON versus CB-nondep versus CB-dep) are presented in Tables 1 and 2, respectively. The separate data from each imaging site are presented in Supplementary Tables S2 and S3.

A subset of matched CON, CB-nondep and CB-dep were selected, to verify volumetric findings. CB-nondep and CB-dep were matched on age, gender, IQ and alcohol, tobacco and cannabis use pattern within each site. This was carried out by first obtaining the mean and standard deviation of each continuous variable of the smallest/reference group (i.e. $\overline{x}_{\mathrm{ref}}$ and σ_{ref}, respectively) by site. Subsequently, each subject's distance score (D) from the reference group, on all variables, was calculated using the equation

$$\mathrm{D} = \sum |(x_v - \overline{x}_{\mathrm{ref.x}})/\sigma_{\mathrm{ref.x}}|$$

where v = the variables: age, IQ, alcohol, tobacco, cannabis onset, cannabis monthly use and cannabis lifetime use. Cannabis-using subjects were ranked and selected by their distance from the reference group. Meanwhile, control subjects were first selected for smoking status, due to the relatively low number of tobacco users in CON relative to CB. Subsequently, the previous equation was applied to select for CON with the lowest distance from the reference group, with regard to age, IQ and alcohol and tobacco use. Nevertheless, we were unable to match CON to CB-nondep and CB-dep on tobacco use, from the Melbourne site. Characteristics and hippocampal volume measures of their matched subset is presented in Table 3 and by imaging site in Supplementary Table S4.

Hippocampal volume comparisons by cannabis use—manual tracing

CON and CB did not differ significantly in right or left hippocampal volume (Table 1).[1] Females had smaller hippocampi compared with males ($F_{1,250}$ = 12.02 and 20.00 for the right and left hippocampus, respectively, $P \leq 0.001$, $\eta_p^2 \geq 0.046$). A site effect was also found ($F_{3,250}$ = 12.34 and 10.65 for the right and left hippocampus, respectively, $P < 0.001$, $\eta_p^2 \geq 0.129$), with participants from Barcelona demonstrating smaller hippocampi than participants from every other site ($P \leq .006$), while

[1] Four CON from the Amsterdam site used between 15 to 50 cannabis joints in their lifetime. When analysis was re-run excluding these subjects, the insignificant group effect remained ($F_{1,246}$ = 2.32 and 1.58, P = 0.13 and 0.21) for the right and left hippocampus, respectively.

Table 1 Sample characteristics and magnetic resonance volumetric measures of controls (CON) and cannabis users (CB) averaged across four sites [mean (SD)].

	CON ($N = 121$)	*CB* ($N = 140$)	t_{259}/χ^2
Age (years)	26.12 (9.03)	28.03 (10.25)	1.58
Gender (% M/F)	70.25/29.75	67.14/32.86	0.29
IQ[a]	109.31 (10.54)	103.45 (10.74)	4.44***
Alcohol (StDr/mth)[b]	19.87 (23.77)	24.43 (25.18)	1.50
Tobacco (Cig/mth)[b]	30.88 (97.92)	254.96 (233.77)	9.82***
Cannabis use			
Age of regular use	-	17.84 (3.38)	-
Current use (cones/month)	-	334.08 (322.32)	-
Lifetime use (cones)	-	57 107 (99 987)	-
Volumetric measures (mm^3)			
Intracranial volume (10^6)	1.55 (0.20)	1.52 (0.17)	1.31
Right hippocampus			
Manual	2584.45 (362.77)	2411.68 (316.24)	2.50[d]
FreeSurfer[c]	4509.66 (469.21)	4381.56 (414.04)	0.00[d]
Left hippocampus			
Manual	2455.05 (342.42)	2314.68 (307.18)	1.56[d]
FreeSurfer[c]	4467.87 (434.48)	4334.63 (434.42)	0.04[d]

[a]Estimated intelligence quotient (IQ) measured with the Dutch version of the National Adult Reading Test (Schmand *et al.* 1991) (Amsterdam), the vocabulary subscale of the Wechsler Adult Intelligence Scale—Third Edition (Wechsler 1997) (Barcelona); the National Adult Reading Test (Nelson 1982) (Wollongong); and the Wechsler Abbreviated Scale of Intelligence (Wechsler 1999) (Melbourne). [b]StDr/mth = standard drinks per month; Cig/mth = cigarettes smoked per month. [c]Two CON subjects were excluded due to poor FreeSurfer hippocampal segmentation (i.e. outlier with hippocampal volume of 2204.48 and 2037.21, respectively), resulting in *n* of CON = 119. [d]*F* statistic for group comparison of hippocampal volume, controlling for imaging site as random factor, gender as fixed factor and ICV, age, IQ and monthly alcohol and tobacco use as covariates. Refer to Supplementary Table S5 for full results. $^*P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.
Hyphen indicates that no data are available/necessary for those places – i.e. non-user controls do not have cannabis use data.

Table 2 Sample characteristics and magnetic resonance volumetric measures of controls (CON), non-dependent (CB-nondep) and dependent (CB-dep) cannabis users averaged across three sites [mean (SD)].

	CON ($N = 106$)	*CB-nondep* ($N = 50$)	*CB-dep* ($N = 70$)	t_{223}/χ^2
Age (years)	24.77 (7.91)	27.07 (10.33)	26.74 (9.18)	1.61
Gender (% M/F)	66.98/33.02	60.00/40.00	64.29/35.71	0.73
IQ[a]	108.65 (10.71)	103.03 (11.13)	102.13 (10.86)	9.15***[d]
Alcohol (StDr/mth)[b]	18.70 (23.90)	21.54 (25.03)	21.88 (22.78)	0.46
Tobacco (Cig/mth)[b]	30.94 (96.72)	236.90 (249.97)	219.72 (197.66)	35.89***[e]
Cannabis use				
Age of regular use	-	17.79 (2.66)	17.44 (3.23)	0.61
Current use (cones/month)	-	229.81 (202.25)	351.64 (290.95)	−2.54*
Lifetime use (cones)	-	32 375 (47 641)	50 431 (72 812)	−1.54
Volumetric measures (mm^3)				
Intracranial volume (10^6)	1.53 (0.19)	1.46 (0.19)	1.53 (0.15)	2.72
Right hippocampus				
Manual	2542.21 (323.36)	2474.32 (326.97)	2340.50 (287.51)	5.91**[f]
FreeSurfer[c]	4476.59 (449.19)	4425.79 (379.48)	4374.22 (422.98)	2.04[f]
Left hippocampus				
Manual	2420.41 (312.78)	2368.56 (329.54)	2250.19 (278.38)	4.49*[f]
FreeSurfer[c]	4453.60 (418.80)	4386.12 (446.93)	4299.16 (422.87)	3.22**[f]

[a]Estimated intelligence quotient (IQ) measured with the Dutch version of the National Adult Reading Test (Schmand *et al.* 1991) (Amsterdam), the vocabulary subscale of the Wechsler Adult Intelligence Scale—Third Edition (Wechsler 1997) (Barcelona); and the Wechsler Abbreviated Scale of Intelligence (Wechsler 1999) (Melbourne). [b]StDr/mth = standard drinks per month; Cig/mth = cigarettes smoked per month. [c]Two CON subjects were excluded due to poor FreeSurfer hippocampal segmentation (i.e. outlier with hippocampal volume of 2204.48 and 2037.21, respectively), resulting in n of CON = 104. [d]CON > CB-nondep, $P = 0.003$; CON > CB-dep, $P < 0.001$. [e]CON < CB-nondep, $P < 0.001$; CON < CB-dep, $P < 0.001$. [f]F statistic for group comparison of hippocampal volume, controlling for imaging site as random factor, gender as fixed factor and ICV, age, IQ and monthly alcohol and tobacco use as covariates. Refer to Supplementary Table S5 for full results. $^*P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.
Hyphen indicates that no data are available/necessary for those places – i.e. non-user controls do not have cannabis use data.

Table 3 Sample characteristics and magnetic resonance volumetric measures of controls (CON), non-dependent (CB-nondep) and dependent (CB-dep) cannabis users in matched subset, averaged across three sites [mean (SD)].

	CON ($N = 41$)	*CB-nondep* ($N = 41$)	*CB-dep* ($N = 41$)	$F_{2,120}/\chi^2$
Age (years)	26.09 (8.68)	28.58 (10.81)	26.71 (8.54)	0.79
Gender (% M/F)	63.4/36.6	63.4/36.6	63.4/36.6	0.00
IQ[a]	107.35 (8.87)	103.33 (12.11)	103.92 (8.78)	1.92
Alcohol (StDr/mth)[b]	24.39 (27.15)	20.65 (22.84)	20.52 (17.22)	0.38
Tobacco (Cig/mth)[b]	76.28 (143.36)	238.83 (253.82)	213.64 (187.22)	7.84**[c]
Cannabis use				
Age of regular use	-	17.82 (2.81)	17.48 (2.58)	0.57
Current use (cones/month)	-	235.40 (209.86)	278.94 (172.76)	1.03
Lifetime use (cones)	-	38 340 (50 702)	37 288 (45 640)	0.10
Volumetric measures (mm^3)				
Intracranial volume (10^6)	1.54 (0.17)	1.49 (0.18)	1.50 (0.17)	0.40
Right hippocampus				
Manual	2525.00 (311.25)	2466.44 (290.37)	2355.56 (310.13)	3.97*[d]
FreeSurfer	4487.90 (451.50)	4454.43 (341.31)	4366.09 (436.79)	1.22[d]
Left hippocampus				
Manual	2373.05 (316.76)	2366.93 (313.91)	2246.15 (287.54)	3.15*[d]
FreeSurfer	4500.68 (458.74)	4413.65 (421.80)	4309.69 (462.13)	1.82[d]

[a]Estimated intelligence quotient (IQ) measured with the Dutch version of the National Adult Reading Test (Schmand *et al.* 1991) (Amsterdam), the vocabulary subscale of the Wechsler Adult Intelligence Scale—Third Edition (Wechsler 1997) (Barcelona); and the Wechsler Abbreviated Scale of Intelligence (Wechsler 1999) (Melbourne). [b]StDr/mth = standard drinks per month; Cig/mth = cigarettes smoked per month. [c]CON < CB-nondep, $P < 0.001$; CON < CB-dep, $P = 0.002$. [d]*F* statistic for group comparison of hippocampal volume, controlling for imaging site as random factor, gender as fixed factor and ICV, age, IQ and monthly alcohol and tobacco use as covariates. Refer to Supplementary Table S5 for full results. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

Hyphen indicates that no data are available/necessary for those places – i.e. non-user controls do not have cannabis use data.

participants from Amsterdam had larger hippocampi than participants from Melbourne ($P \leq 0.018$). IQ significantly affected left hippocampus volume ($F_{1,250} = 4.33$, $P = 0.039$, $\eta_p^2 = 0.017$). None of the other covariates (i.e. age, IQ, alcohol use and tobacco use) were statistically significant in the model ($P \geq 0.054$, $\eta_p^2 \leq 0.015$).

Hippocampal volume comparisons by cannabis dependence—manual tracing

Analyses comparing CON, CB-nondep and CB-dep (from three sites) revealed a significant effect of dependence group in the right ($F_{2,215} = 5.91$, $P = 0.003$, $\eta_p^2 = 0.052$, medium-effect size) and left ($F_{2,215} = 4.49$, $P = 0.012$, $\eta_p^2 = 0.040$, medium-effect size) hemisphere (Table 2).[2] CB-dep had significantly smaller right and left hippocampi compared with both CON ($P = 0.003$ and 0.008) and CB-nondep ($P = 0.006$ and 0.016) (Fig. 1). As in the four-site analysis, gender ($F_{1,215} = 16.39$ and 27.57 for right and left hippocampus, respectively, $P < 0.001$) and site effects ($F_{2,215} = 19.18$ and 15.89 for right and left hippocampus, respectively, $P < 0.001$) were significant. Females had smaller hippocampi than males, and again, participants from the Barcelona site had smaller hippocampi than those from the other two sites ($P \leq 0.001$), while participants from the Amsterdam site had larger hippocampi than those from Melbourne ($P \leq 0.026$). None of the covariates were statistically significant in the model ($P \geq 0.087$, $\eta_p^2 \leq 0.014$).

To establish the specificity of volumetric differences to cannabis dependence rather than cannabis use or exposure (and particularly because CB-dep had significantly greater monthly use than CB-nondep), CB-dep and CB-nondep were further compared, additionally controlling for cannabis use measures (current monthly cones, lifetime cones and age of regular use). The significant group difference persisted, with CB-dep showing smaller right ($F_{1,104} = 6.02$, $P = 0.016$, $\eta_p^2 = 0.055$, medium-effect size) and left hippocampi ($F_{1,104} = 6.19$, $P = 0.014$, $\eta_p^2 = 0.056$, medium-effect size) than CB-nondep after controlling for these cannabis use measures.

Hippocampal volume comparisons by cannabis dependence in matched subset—manual tracing

Finally, the subset of matched CON, CB-nondep and CB-dep were compared on hippocampal volume. Gender distribution, age, IQ, alcohol use and tobacco use were matched across groups within each site, apart from

[2] Four CON from the Amsterdam site used between 15 to 50 cannabis joints in their lifetime. When analysis was re-run excluding these subjects, the significant dependence effect remained ($F_{2,211} = 5.72$ and 4.41, $P = 0.004$ and 0.013) for the right and left hippocampus, respectively.

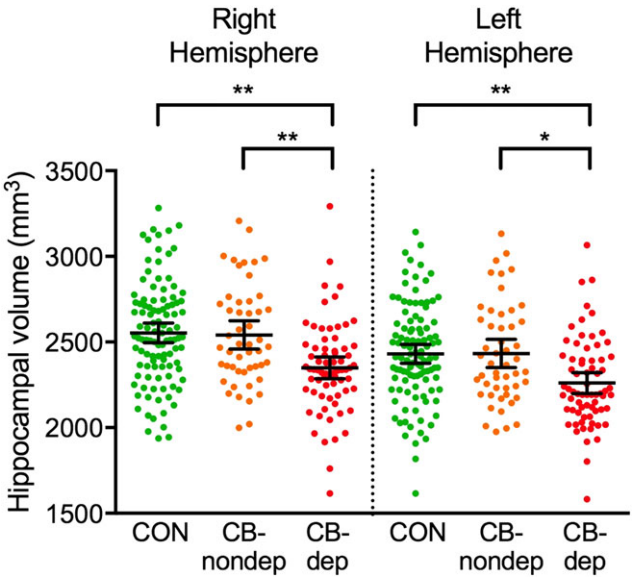

Figure 1 Right and left hippocampal volume in controls (CON), non-dependent (CB-nondep) and dependent (CB-dep) cannabis users, corrected for intracranial volume and gender; bars represent 95% confidence interval; $^{*}P < 0.05$ $^{**}P < 0.01$

Melbourne, for which we were unable to match tobacco use. Furthermore, CB-nondep and CB-dep were matched on all previously mentioned variables (i.e. gender, age, IQ and alcohol and tobacco use) and cannabis use pattern. The effect of cannabis dependence persisted for the right ($F_{1,112} = 3.97$, $P = 0.022$, $\eta_p^2 = 0.066$) and left hippocampi ($F_{1,112} = 3.15$, $P = 0.047$, $\eta_p^2 = 0.053$). CB-dep users demonstrated significantly smaller hippocampi than CB-nondep users in both hemispheres ($P = 0.016$ and $P = 0.022$, respectively) and a smaller right hippocampus than CON ($P = 0.020$).

Hippocampal shape comparisons by cannabis use and dependence—manual tracing

Cluster-based shape analysis was performed controlling for ICV, imaging site, gender, IQ, age, alcohol use and tobacco use. Comparison between (1) CON and CB revealed no significant shape difference between groups. However, comparison between (2) CON, CB-nondep and CB-dep demonstrated a significant shape difference between CB-nondep and CB-dep in the right and left hippocampus (Fig. 2a, d) but not between CON and CB-nondep or CON and CB-dep.[3] Specifically, deflation occurred along the superior-medial body of the hippocampi of CB-dep relative to CB-nondep. Nevertheless, when comparison was performed between the subset of (3) matched CON, CB-nondep and CB-dep, deflation in CB-dep relative to CB-nondep did not survive family-wise error (FWE)-correction across the image space.

[3] Four CON from the Amsterdam site used between 15 to 50 cannabis joints in their lifetime. When analysis was re-run excluding these subjects, results remained similar.

Hippocampal volume and shape—FreeSurfer versus manual tracing

All hippocampal volume and shape analyses were replicated using the automated segmentation software FreeSurfer. FreeSurfer performance was also validated, relative to manual tracing, by examining the correlation between both methods, and the percent volume overlap. Results for FreeSurfer-segmented hippocampal comparison between (1) CON and CB, (2a) CON, CB-nondep and CB-dep, (2b) CB-nondep and CB-dep only, and (3) matched subset of CON, CB-nondep and CB-dep are presented in Supplementary Table S5. Briefly, there was no significant hippocampal volume difference between CON and CB, but CB-dep users similarly showed a smaller left hippocampus than CB-nondep users ($P = 0.013$). When only CB-nondep and CB-dep users were compared, additionally controlling for cannabis use pattern, CB-dep users again demonstrated significantly smaller right and left hippocampi relative to CB-nondep users ($P = 0.027$ and $P = 0.005$, respectively). When the matched subset of CON, CB-nondep and CB-dep were compared, however, no significant dependence effect was found. Cluster-based shape analysis of FreeSurfer-segmented hippocampi meanwhile only demonstrated a shape difference between CB-dep and CB-nondep users that did not survive FWE-correction. While the FreeSurfer-segmented hippocampi were strongly correlated with the manual tracing ($R = 0.72$ and 0.66 for the right and left hippocampus, respectively, $P < 0.001$), the FreeSurfer hippocampi are systematically larger than the manual output, as illustrated in the Bland–Altman plot (Supplementary Fig. S3). Estimation of volume overlap between both methods suggests an average volume overlap of 71.2 percent ($SD = 4.39$ percent) and 70.10 percent ($SD = 4.75$ percent) for the right and left hippocampus, respectively.

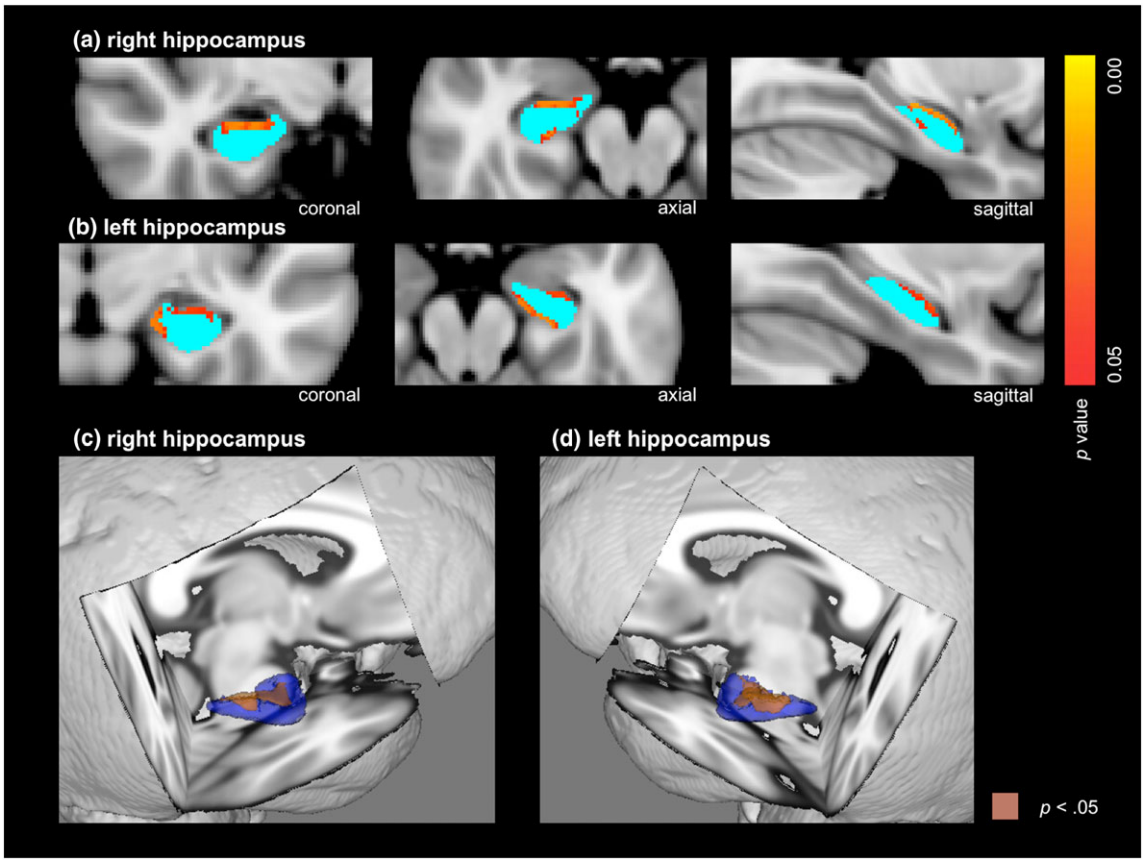

Figure 2 (a, b) Cross-sectional coronal, axial and sagittal slices of magnetic resonance scans and (c, d) 3D rendering of the right and left hippocampus, depicting areas of deflation in hippocampal shape in CB-dep compared with CB-nondep users

The larger hippocampal volume produced by FreeSurfer may be due to its greater tendency to include surrounding structures and cerebrospinal fluid, as illustrated in example slices in Supplementary Fig. S3.

DISCUSSION

In this large-scale multi-site study, we demonstrated significant hippocampal volume reduction only in cannabis dependent users relative to both non-user CON and non-dependent users, irrespective of extent of cannabis use. Shape difference was also observed in the right and left hippocampus, only in dependent users (deflation of the superior-medial body) relative to non-dependent users. These results suggest that hippocampal volume and shape alterations may be specific to cannabis dependence rather than non-dependent regular cannabis use. Our findings are consistent with previous work reflecting dependence-specific effects, e.g. in neuroanatomical and functional alteration across the cortical and limbic regions (Filbey & Dunlop 2014; Chye *et al.* 2017a; Chye *et al.* 2017b). Future investigative efforts should thus be mindful in assessing and discriminating between cannabis use and dependence when evaluating the harms and vulnerabilities associated with chronic cannabis use.

Hippocampal volumetric reduction is the most consistently reported neuroanatomical finding in regular cannabis users relative to non-users (Rocchetti *et al.* 2013; Koenders *et al.* 2016; Yücel *et al.* 2016; Lorenzetti *et al.* 2016c; Chye *et al.* 2017b) but was not observed in all studies (e.g. Tzilos *et al.* 2005; Medina *et al.* 2007; Gilman *et al.* 2014; Mashhoon *et al.* 2015; Weiland *et al.* 2015). We were well-powered to detect group differences in a large sample (aggregated across well-controlled studies from four international research sites) and found no volume reduction in cannabis use *per se* but specifically in dependent users. Notably, these findings were not driven by cannabis use level (i.e. monthly or lifetime use), suggesting cannabis dependence-specific effects on hippocampal morphology to be dissociated from those due to level of cannabis use. This contrasts previous reports of a dose-dependent association between hippocampal volume and cannabis dosage (Yücel *et al.* 2008; Ashtari *et al.* 2011; Cousijn *et al.* 2012). However, none of the aforementioned studies discriminated between dependent and non-dependent users in their samples and might not have been able to dissociate hippocampal differences linked to dosage versus dependence. Indeed, less than 40 percent of frequent cannabis users (i.e. using ≥3 days/week for ≥1 year) will develop a dependence

syndrome, irrespective of level of use (van der Pol *et al.* 2013c). This distinction in the cannabis user population (i.e. dependence versus non-dependence) may explain why a number of studies have failed to detect hippocampal volume differences in cannabis users compared with CON, as these studies may have included varying proportions of dependent and non-dependent users (Tzilos *et al.* 2005; Medina *et al.* 2007; Gilman *et al.* 2014; Mashhoon *et al.* 2015; Weiland *et al.* 2015).

We also found a localized shape difference between dependent and non-dependent users along the superior-medial body of the hippocampus, roughly coinciding with the cornu ammonis and dentate gyrus (CA3 and CA4/DG) regions (Finegersh *et al.* 2011). While this result did not survive FWE correction in the subset of users matched on age, IQ and substance use, it is possible that the smaller sample (i.e. from 226 in the original analysis to 123 in this analysis) resulted in reduced power to detect subtle shape effects. Hippocampal shape alterations in cannabis users have only been examined in four prior studies, demonstrating regional shape differences in current users, recreational users (*Mdn* = 6–10 lifetime use) and users with a past cannabis use disorder (Solowij *et al.* 2013; Smith *et al.* 2015; Orr, Paschall, & Banich 2016; Koenders *et al.* 2017). Our finding is consistent with previous reported alterations in regular cannabis users [i.e. shape deflation along the hippocampal head and body (Solowij *et al.* 2013; Koenders *et al.* 2017)] and in dependent users [i.e., reduced CA3 and CA4/DG volume (Chye *et al.* 2017b)]. Deflation confined to the CA3 and CA4/DG hippocampal subregions is noteworthy as these are the major sites for adult neurogenesis and subsequent innervation of new neurons, a process crucial for learning and memory, as well as affective and stress regulation (Canales 2007; Chambers 2013). Indeed, poorer cognitive and emotive functioning are documented in dependent cannabis users (Solowij & Battisti 2008; van der Pol *et al.* 2013a; Broyd *et al.* 2016). We were unfortunately unable to explore whether cannabis dependence-related hippocampal morphology mediates differences in cognitive and emotive functioning (e.g. depressive or anxiety symptoms) in the current study. Such knowledge may be useful for understanding the interaction between cannabis dependence and functioning in relation to brain structure and presents a potential avenue for future work.

Prominent theories of addiction propose that vulnerabilities in the decision-making process coupled with distress associated with negative mood states are the key drivers in persistent substance taking observed in substance dependence (Koob 2008; Koob & Le Moal 2008; Redish, Jensen, & Johnson 2008; Volkow & Morales 2015). Our finding of hippocampal alteration specific to cannabis dependence supports theories suggesting dependence-specific neuroalterations. The amygdala–hippocampal system is involved in affective processing (Ekhtiari, Victor, & Paulus 2017), with impaired hippocampal functioning (e.g. low hippocampal neurogenesis) further linked to poor stress regulation (Hyman & Sinha 2009; Schloesser, Manji, & Martinowich 2009). Increased stress reactivity and negative mood state pose a vulnerability factor which is strongly associated with dependence in cannabis users, beyond and distinct from extent of cannabis use (Stewart 2003; Koob 2009; van der Pol *et al.* 2013c). Additionally, hippocampal function is also necessary to guide learning and adaptive behavior, with impaired function suggested to restrict the complexity and flexibility of motivational learning that subserves the extinction of substance use behavior, thus contributing to the maintenance of dependence (Canales 2007; Chambers, Bickel, & Potenza 2007; Redish *et al.* 2008; Chambers 2013). While future efforts are necessary to expand on the link between hippocampal neuroanatomy and the cognitive, stress and affective regulation process guiding cannabis dependence, it nonetheless appears that dependent cannabis users may be distinctly impacted in neuroanatomy (Filbey & Yezhuvath 2013; Chye *et al.* 2017a; Chye *et al.* 2017b).

Finally, we compared the consistency between two separate methods of measuring hippocampal volumes, i.e. FreeSurfer and manual segmentation (refer to Supporting Information), and showed that these were highly correlated (about 70 percent volume overlap). FreeSurfer produced systematically larger hippocampi than did manual segmentation, which may be due to its greater tendency to include surrounding structures and cerebrospinal fluid (refer to the example slices in Supplementary Fig. S3). However, we still found a significant effect of cannabis dependence in FreeSurfer-segmented hippocampi (i.e. in analyses controlling for cannabis use pattern but not in the matched subset analyses, Supplementary Table S5), suggesting mostly consistent results from both methods. The manual segmentation method is considered the gold standard for evaluating hippocampal volume (Velakoulis *et al.* 1999) and assumed to be superior to automated methods (i.e. SPM, FSL and FreeSurfer), as it allows for a more fine-grained inspection of hippocampal volume and shape. Meanwhile, FreeSurfer's estimations of hippocampal volume tend to show a larger variance, in addition to a tendency to underestimate gray matter volume with increasing scanner noise, causing its output to be more subject to hardware-related differences (Butts 2013; Wenger *et al.* 2014; Fellhauer *et al.* 2015). As such, when assessing impacts on the morphology of the hippocampus, it may be preferable for studies to adopt manual tracing methods wherever feasible.

Some limitations of this study must be addressed. Collating a mega-analysis from multiple imaging sites meant that site-related factors such as scanner differences and geographical differences could have confounded the results. To address this, we controlled for imaging site in all our group analyses. Furthermore, the hippocampal volume of cannabis dependent users was clearly reduced relative to non-dependent users and CON at every site, suggesting that no single site was driving the observed results (Supplementary Fig. S2). Secondly, the cross-sectional nature of our analysis precludes interpretation on the causality of the effects, i.e. whether altered hippocampal morphology pre-exists or is consequent to cannabis use and dependence. Finally, as different imaging sites have adopted different instruments in measuring cannabis dependence, we could not directly compare levels of dependence severity with hippocampal morphology across sites or examine severity in regression models. Instead, we adopted validated cut-offs (Lecrubier *et al.* 1997; Swift, Copeland, & Hall 1998; van der Pol, *et al.* 2013b) to consistently investigate hippocampal morphology between dependent and non-dependent users. Studies using consistent diagnostic instruments of substance use disorders (e.g. DSM-5; American Psychiatric Association 2013) are needed to verify the association between hippocampal morphology and dependence severity, particularly in further delineating the relationship between dependence, cognitive and affective regulation and the neuroanatomy of substance users (Solowij, Lorenzetti, & Yücel 2016; Lorenzetti *et al.* 2016a; Lorenzetti *et al.* 2016b; Lorenzetti *et al.* 2016c).

CONCLUSION

We extend on previous studies of hippocampal morphological alteration (i.e. shape and volume) in non-dependent and dependent cannabis users in a large multi-site imaging cohort, using both manual tracing and automated methods. Hippocampal volume reduction was specific to dependent users, even after controlling for cannabis dosage and sample characteristic (i.e. age, IQ and alcohol and tobacco use). There was also an emerging shape difference along the superior-medial boundary of the hippocampus, between dependent and non-dependent users. Our findings suggest that not all cannabis users are alike, with a subgroup of vulnerable users—dependent users—showing hippocampal morphological alterations compared with non-dependent users and CON. Further steps should be made to characterize and verify the neural and behavioral differences that separate non-dependent and dependent cannabis users in large normative samples and treatment-seeking populations, whether as vulnerability factors or consequent of use, to better identify and pre-empt the transition of cannabis users to dependence.

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CONFLICT OF INTEREST

All authors report no financial interest or potential conflict of interest.

FINANCIAL DISCLOSURES

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AUTHORS CONTRIBUTION

Y. C., V. L., C. S., M. Y. and N. S. were responsible for the study concept and design. V. L., A. B., J. C., A. E. G., R. M. S., S. W., M. Y. and N. S. contributed to the acquisition of data. Y. C. performed the data analysis, and V. L., C. S. and M. J. assisted in analysis methodology. Y. C. drafted the manuscript. V. L., C. S., M. Y. and N. S. provided critical intellectual input and revision to the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Assessment instruments by imaging site

Table S2. Sample characteristics and MR volumetric measures of controls (CON) and cannabis users (CB) at each site (mean (SD))

Table S3. Sample characteristics and MR volumetric measures of controls (CON), non-dependent (CB-nondep) and dependent (CB-dep) cannabis users at each site where dependence measures were available (mean (SD))

Table S4. Sample characteristics and MR volumetric measures in matched subset of controls (CON), non-dependent (CB-nondep) and dependent (CB-dep) cannabis users at each site where dependence measures were available (mean (SD))

Table S5. Group comparison results of right and left hippocampal volume, with both manual tracing method and automated FreeSurfer method

Figure S1. Flow chart of hippocampal shape analysis steps, using the Oxford Centre for Functional MRI of the Brain (FMRIB) Software Library (FSL). **(a, blue)** transform hippocampal object maps (ROI) to standard (MNI)

space; **(b, orange)** average all hippocampal objects (ROI) to form an average boundary (ROI_{Bound}); **(c, green)** separately binarise each hippocampal object (ROI_{Bin}); **(d, purple)** calculate signed distance (Dis) between binarised hippocampal object maps and average boundary**; (e, red)** run group comparison on distance maps. T1 = T1-weighted MRI, subscript number indicates subjects' ID, DOF = degree of freedom

Figure S2. Average hippocampal volume (collapsed across hemisphere) in controls (CON), non-dependent (CB-nondep) and dependent (CB-dep) cannabis users, separated by imaging site, and corrected for intracranial volume (ICV) and gender; bars represent 95% confidence interval. * *p<.05;* ** *p<.01,* *** *p<.001*

Figure S3. (A) Cross-sectional coronal, axial and sagittal slices of an example MR scan with overlay of FreeSurfer and manual hippocampus, and **(B,C)** Bland-Altman mean-difference plots for right and left hippocampal volumes.