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Published in:
Journal of Psychopharmacology

DOI:
10.1177/0269881117718380

Original Paper

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Journal of Psychopharmacology
2017, Vol. 31(8) 1027–1034

DOI: 10.1177/0269881117718380
journals.sagepub.com/home/jop

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Abstract

Background: Cannabis exposure, particularly heavy cannabis use, has been associated with neuroanatomical alterations in regions rich with cannabinoid receptors such as the hippocampus in some but not in other (mainly cross-sectional) studies. However, it remains unclear whether continued heavy cannabis use alters hippocampal volume, and whether an earlier age of onset and/or a higher dosage exacerbate these changes.

Methods: Twenty heavy cannabis users (mean age 21 years, range 18–24 years) and 23 matched non-cannabis using healthy controls were submitted to a comprehensive psychological assessment and magnetic resonance imaging scan at baseline and at follow-up (average of 39 months post-baseline; standard deviation=2.4). Cannabis users started smoking around 16 years and smoked on average five days per week. A novel aspect of the current study is that hippocampal volume estimates were obtained from manual tracing the hippocampus on T1-weighted anatomical magnetic resonance imaging scans, using a previously validated protocol.

Results: Compared to controls, cannabis users did not show hippocampal volume alterations at either baseline or follow-up. Hippocampal volumes increased over time in both cannabis users and controls, following similar trajectories of increase. Cannabis dose and age of onset of cannabis use did not affect hippocampal volumes.

Conclusions: Continued heavy cannabis use did not affect hippocampal neuroanatomical changes in early adulthood. This contrasts with prior evidence on alterations in this region in samples of older adult cannabis users. In young adults using cannabis at this level, cannabis use may not be heavy enough to affect hippocampal neuroanatomy.

Keywords

Cannabis, marijuana, longitudinal, hippocampus, manual tracing, medial temporal lobe, structural MRI

Introduction

Cannabis is frequently used and is associated with adverse outcomes on mental health (Degenhardt and Hall, 2012; Hall and Degenhardt, 2009). The frequency of cannabis use typically peaks in young adulthood (Copeland et al., 2013), a period of extensive neuroanatomical remodelling (Ostby et al., 2009; Raznahan et al., 2014), particularly in areas high in cannabinoid receptors via which cannabinoid compounds exert their effects (Jacobus and Tapert, 2014). The primary psychoactive cannabinoid compound tetrahydrocannabinol (THC) is found in increasing levels in commonly available cannabis (United Nations Office on Drugs and Crime, 2014) and may be neurotoxic (Chan et al., 1998; Rocchetti et al., 2013). Young adults who use cannabis on a regular basis might be particularly sensitive to the potential neurotoxic effects of cannabinoid exposure (Jacobus and Tapert, 2014).

The hippocampus is a brain region with one of the highest densities of cannabinoid receptors (Glass et al., 1997), and may thus be particularly vulnerable to the effects of cannabinoid exposure (Lorenzetti et al., 2013; Ranganathan and D'Souza, 2006). Hippocampal volumetric reductions have been reported in cannabis users (for a meta-analysis, see Rocchetti et al., 2013), although only for the left and right hemisphere combined. In our previous study in the same sample (Koenders et al., 2016) using

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voxel-based morphometry (VBM), we found that grey matter development of the hippocampal region over a period of 39 months did not differ between cannabis users aged 21–25 years and age-matched controls. Importantly, at baseline there was a significant negative correlation between the amount of use in grams per week and grey matter density in a cluster comprising the left hippocampus. However, VBM may suffer from bias due to suboptimal and limited localization accuracy (Mechelli et al., 2005). In addition, global measures of volume lack information on the details of the topography within a given brain structure (Gilman et al., 2014). We decided to study the hippocampal region in more detail using manual tracing of the hippocampus in the same sample. The use of a manual tracing technique by an experienced tracer has the benefit of enhanced sensitivity to subtle effects in hippocampal shape and greater precision in detecting inter-individual variability in anatomical boundaries when compared to the application of automated protocols such as VBM (Stjepanovic et al., 2013). Manual tracing also allows shape analyses, enabling examination of whether cannabis use affects specific hippocampal sub regions (Gilman et al., 2014; Gogtay et al., 2006).

During normal development hippocampal volumes develop in a curvilinear slope, first increasing with age (Goddings et al., 2014; Ostby et al., 2009) and decreasing around the mid 30s (Ostby et al., 2009; Raz et al., 2004). Continued cannabis use during young adulthood may influence development through causing hippocampal volumetric reductions in cannabis users over time.

Small hippocampal volumes have been found most consistently in samples with high levels of cannabis exposure (for reviews, see Lorenzetti et al., 2013; Rocchetti et al., 2013). Also, an earlier age of onset of cannabis use may have a negative effect on hippocampal structure and function (Solowij and Battisti, 2008). However, in the meta-analysis of Rocchetti et al. (2013), no correlation was found between duration of use and hippocampal volumes. Also, cross-sectional structural neuroimaging studies to date did not find associations between hippocampal volume and age of onset in cannabis users (for a review, see Lorenzetti et al., 2013). An earlier age of onset of cannabis use was found to be related to an elevated risk for cannabis dependence (Grant and Pickering, 1998) and development of psychotic disorders (Arseneault et al., 2002). Also, an earlier age of onset of cannabis use was shown to be related to impaired performance on hippocampal-mediated cognitive tasks like the spatial working memory task (Harvery et al., 2007) and the Rey Auditory Verbal Learning Test (Harvery et al., 2007). Therefore, we propose that an earlier age of onset may hamper the typical developmental increase (Goddings et al., 2014; Ostby et al., 2009) in hippocampal volume in cannabis users.

To evaluate this hypothesis we conducted a longitudinal study with a precise and reliable assessment of hippocampal volume and (also for the first time) hippocampal shape, using manual tracing of hippocampal neuroanatomy in heavy cannabis using young adults and matched healthy non-using controls. Participants were examined at baseline (BL) and at three-year follow-up (FU). We expected that (a) heavy cannabis users would show reduced hippocampal volumetric gain over time (Goddings et al., 2014; Ostby et al., 2009; Raz et al., 2004); (b) earlier onset of heavy cannabis use and (cumulative) dose would be associated with a larger decrease in normally expected gains in hippocampal volume in cannabis users.

Methods

This study was nested within a three-year longitudinal magnetic resonance imaging (MRI) investigation of heavy cannabis users (Cousijn et al., 2014). Cannabis users and controls underwent a comprehensive psychological assessment and MRI scan BL in 2009 and at FU in 2012, after an average of 39 months (standard deviation (SD) 2.4 months). The medical ethics committee of the Academic Medical Centre approved the study and all participants signed informed consent before participation.

Participants

At baseline, we recruited 33 heavy cannabis users and 43 controls, aged 18–25 years, through advertisements on the Internet and in cannabis outlets (coffee-shops). Both groups were matched for age, gender, education, estimated intelligence (Dutch Adult Reading Test (DART); Schmand et al., 1991), and alcohol use (Alcohol Use Disorder Identification Test (AUDIT); Saunders et al., 1993). Cannabis users were included if they used cannabis for at least two years for more than 10 days per month and did not seek treatment for cannabis use problems. All cannabis users included in this study smoked cannabis joints. Controls were current non-users, who used cannabis on less than 50 occasions during their lifetime and did not use cannabis during the past year. All participants were instructed to abstain from alcohol and drugs 24 h prior to participation. We performed urine toxicology tests at both assessments to corroborate self-reported substance use (Roese and Jamieson, 1993). Other exclusion criteria were general MRI-contraindications, major physical disorders, and psychiatric disorders (including the presence of any psychotic symptoms), which were assessed with the Mini-International Neuropsychiatric Interview (MINI; Dutch version 5.0.0, Sheehan et al., 1998). Participants were financially compensated for taking part in the study.

Of the 33 cannabis users recruited for BL assessment, 24 users completed the FU assessment. Twenty-seven of the 40 controls assessed at BL completed the FU assessment. We excluded three cannabis users that quit cannabis use between BL and FU assessment and three controls that started cannabis use over the same period. In addition, we excluded two participants with low quality MRI scans (one cannabis user and one control). The group available for analysis thus consists of 20 heavy cannabis users and 23 controls.

Questionnaires

Participants underwent a comprehensive assessment of history of cannabis use, from which we derived age of onset of regular (weekly) use and lifetime cumulative dosage, measured in ‘amount of grams of cannabis’. The latter was calculated at baseline as (g per week)×52 (weeks per year), multiplied by the amount of years of regular (i.e. weekly) use (adopted from Yücel et al., 2008). We also computed the change of use in the interval between BL and FU (in g), and accounted for periods of lighter and heavier use.

Problem severity and frequency of cannabis use were measured using the Cannabis Use Disorder Identification Test (CUDIT; Adamson and Sellman, 2003). We assessed the severity

of nicotine dependence using the Fagerström Tolerance Questionnaire (FTQ; Fagerstrom and Schneider, 1989). At follow-up, the MINI (Dutch version 5.0.0, Sheehan et al., 1998) was conducted by two experienced psychologists (LK and WAMV) to assess the presence of DSM-IV mental disorders.

MRI acquisition and processing

T1-weighted structural MRI scans were acquired from all participants on a 3T MRI scanner (Intera, Philips Healthcare, Best, The Netherlands) with a phased array SENSE eight-channel receiver head coil (T1 turbo field echo, TR 9.6 s, TE 4.6 s, 182 slices, slice thickness 1.2 mm, FOV 256×256, in-plane resolution 256×256 mm, flip angle 8°). Images were visually inspected for artifacts, and subsequently skull stripped of non-brain tissue using BET (Smith, 2002) and intensity inhomogeneities were corrected for using Statistical Parametric Mapping 8 software (SPM8; http://www.fil.ion.ucl.ac.uk/spm). Images were linearly registered to the standard template of the Montréal Neurological Institute (MNI) with six degrees-of-freedom rigid body transformations using FLIRT (Jenkinson and Smith, 2001). Subsequently, voxel dimension drift was corrected by registering follow-up to baseline images with nine degrees of freedom (Whitwell et al., 2004), also using FLIRT (Jenkinson and Smith, 2001).

Tracing was performed blind to group membership and time of assessment by the first author (LK) using ANALYZE version 11 (Mayo Clinic, Rochester, USA). The hippocampus was defined based on a previously validated technique and proceeded from the caudal (i.e. tail) to the rostral end of the hippocampus (i.e. head) on coronally displayed MRI slices (Velakoulis et al., 1999; Velakoulis et al., 2006; Whittle et al., 2008). Hippocampal tracings included the hippocampus proper, the dentate gyrus, the subiculum, and part of the fimbria and alveus. For a detailed tracing protocol, see Supplementary Material, Section 1.

Reliability of hippocampal tracing was assessed by comparing ten randomly selected images with those of an experienced tracer (VL). Absolute agreement inter-rater reliability scores for left and right hemisphere were ICC=0.92 and ICC=0.97, respectively, and intra-rater reliability for left and right hemisphere were 0.98 and 0.94, respectively.

Volumetric estimates were obtained by summing all voxels within traced brain regions on consecutive coronal slices. Hippocampal volumes were adjusted for intracranial volume (ICV) by an analysis of covariance approach:

Adjusted volume=raw volume–b×(ICV–mean ICV)

where b is the slope of the regression line of hippocampal volume (left or right) and ICV (BL or FU) respectively (Erickson et al., 2011). Adjusted volumes were used for all analyses described in this article.

Shape analyses

To investigate specific morphometric changes within the hippocampus, we performed a hippocampal shape analysis through the University of North Carolina shape analysis toolkit, version 1.12 (Spherical Harmonic Shape Description (SHPARM-PDM); Brechbühler et al., 1995). A detailed description of the methodology is available in Styner et al. (2004, 2006). In brief, images of segmented hippocampi were first converted to surface meshes, and a spherical parameterization was computed, creating a one-to-one mapping between surface and sphere. The surface was expanded into a series of spherical harmonics, truncated at a degree of k=15. The coefficients of the series expansion were normalized in order to make them invariant to rotation, translation and scale. The SPHARM parameterization was transformed into a triangulated surface (called the SPHARM-PDM), based on a uniform subdivision of the spherical parameterization. Each hippocampus was described by a set of n=1002 landmarks. The SPHARM-PDM was spatially aligned using rigid Procrustes alignment, giving a one-to-one mapping between surface points of each pair of hippocampi. Finally, to test longitudinal shape changing, MeshMath was applied to compute the distance map (with information of both length and direction at each landmark) between the two time points.

Statistical analyses

Demographic data were compared between groups and over time using repeated measures analysis of variance tests, independent samples t-tests and paired samples t-tests. To assess whether cannabis use was related to changes in hippocampal volumes over time, we performed a repeated measures analysis of covariance (ANCOVA), with hippocampal volumes as dependent variables, time of assessment and hemisphere as repeated measure, group as between subjects factor, and gender and age (measured at baseline centred around the grand mean) as covariates.

To examine whether age of onset of regular cannabis use and levels of cannabis exposure mediate changes in hippocampal volumes over time, we performed a repeated measures ANCOVA within the cannabis group with hippocampal volume as dependent variable and the median split of cumulative dosage at baseline as a covariate: a low cumulative dose (n=9, <351 g) and a high cumulative dose subgroup (n=10, >351 g). To examine the 'independent' impact of cumulative dose (both of pre-baseline period and the time to follow-up, i.e. dosage change) versus age of onset, we used the following statistical model: time of assessment and hemisphere were entered as repeated measures, median split cumulative dosage (i.e. high vs low) as between subjects factor, and age, age of onset of regular use, dosage change and gender as covariates. We repeated all analysis without the subjects that started using drugs other than cannabis or developed a psychiatric disorder to control for these confounding factors. For an overview of the sample's psychiatric comorbidities and/or polysubstance use see Supplementary Material, Table 6.

We used Shape Analysis MANCOVA from the SPHARM-PDM toolbox for the shape analyses, for left and right hemisphere separately. All results were adjusted for age at baseline, gender and intracranial volume. Due to failed preprocessing we had to exclude some tracings. For bilateral hippocampi we excluded five subjects (left: two cannabis users, three healthy controls (HCs); right: three cannabis users, one HC). As dependent variables we used the individual distance maps, containing the difference between FU and BL per surface point (1002 landmarks). We used a MANCOVA for the between-group analyses (i.e. group and dosage), and the toolkit's correlational analysis to calculate Spearman's correlations with age of onset. Further, a one-sample t-test was applied to check the time effect on hippocampal shape changing across whole group. The output was controlled for multiple comparisons using the false discovery rate (FDR) correction procedure (Paniagua et al., 2009; Pantazis

Table 1. Demographic information at each time point. There were no significant interaction effects between time and group, therefore only main effects are reported.

	CB (*n*=20)		HC (*n*=23)		Main effects	
	Baseline	Follow-up	Baseline	Follow-up	F_{time}	F_{group}
Age	20.64 (2.23)	24.16 (2.59)	21.79 (2.6)	25.07 (2.64)	829.04[a]	1.83
DART	105 (5.45)	106 (5.00)	106 (5.65)	104 (9.42)	0.57	0.01
CUDIT	13.3 (6.59)	13.5 (8.25)	0.04 (0.21)	0.3 (0.70)	0.10	92.76[a]
AUDIT	6.10 (3.39)	8.25 (5.11)	4.74 (3.73)	5.96 (3.38)	9.38[b]	3.06
FTQ	2.75 (2.40)	3.45 (2.80)	0.52 (1.16)	0.83 (1.59)	4.29[b]	17.67[a]
Daily cigarettes	7.08 (7.37)	8.31 (9.02)	1.35 (3.16)	1.45 (5.25)	0.45	13.76[a]
Onset nicotine use, age years[c]	–	13.53 (4)	–	16.63 (3.70)	n/a	$t(21)=-1.90$
Cannabis 1st use, age years[d]	–	14.4 (1.47)	–	18.21 (3.02)	n/a	$t(18)=-4.38$[a]
Onset regular use, age years	–	16.15 (2.32)	–	n/a	n/a	n/a
Cumulative dosage, g lifetime	655 (565)	1220 (812)	n/a	n/a	$t(18)=-4.81$[b]	n/a
Smoking, g per week	2.75 (1.80)	3.44 (3.29)	n/a	n/a	$t(19)=-0.93$	n/a
Smoking, days per week	4.65 (1.59)	4.95 (2.31)	n/a	n/a	$t(19)=-0.64$	n/a
ICV	1389 (129)	1455 (140)	1438 (135)	1490 (145)	50.30[a]	1.03

AUDIT: Alcohol Use Disorder Identification Test; CB: cannabis group; CUDIT: Cannabis Use Disorder Identification Test; DART: Dutch Adult Reading Test; FTQ: Fagerström Tolerance Questionnaire; HC: healthy control group; ICV: intracranial volume; n/a: not applicable.
F_{time} refers to the main effect of time; F_{group} refers to the main effect of group, i.e. the difference between the two groups.
[a]$p<0.01$; [b]$p<0.05$; [c]CB=15, HC=8; [d]CB=20, HC=14.

et al., 2004; Styner et al., 2004). Results were visualized for the left and right hemisphere on a mean surface of all tracings, using KWVisu (Oguz et al., 2006).

Results

Sample characteristics

Sample demographics, clinical, neurocognitive and substance use characteristics are provided in Table 1. The gender distribution was similar between groups (14 males, six females) and the control group (13 males, 10 females; $\chi^2(1)=0.83$, $p=0.36$).

Cannabis users and controls did not significantly differ on premorbid IQ (DART) and alcohol dependence (AUDIT), while onset of nicotine use showed a statistical trend. Cannabis users showed higher levels of cannabis and nicotine dependence than controls (CUDIT and FTQ scores, respectively) at BL and FU. CUDIT scores remained stable over time, but FTQ scores increased between BL and FU up in both groups. The amount of daily cigarettes and alcohol use problems was stable over time in both groups. We found an increase over time in weekly dose of cannabis use and also in number of days of use, at a trend-level significance (Table 1). The dosage change of cannabis use between BL and FU ranged between 31–1872 g (M=564 g; SD=511 g). Comorbid DSM-IV diagnoses assessed at FU are reported in Supplementary Material, Table 6.

All participants but one remained abstinent for one or two days before the assessment at both BL (less than a day, *n*=1; 1 day, *n*=11; 2 days or more, *n*=8) and FU (less than a day, *n*=1; 1 day, *n*=11; 2 days or more, *n*=8). Control participants used cannabis on an average of two occasions in their lifetime on the baseline assessment (range 0–30) and on four occasions (range 0–25) on the follow-up assessment. One control subject used cannabis on 30 occasions. This subject was comparable to the control group on all other behavioural clinical substance use measures on visual data inspection. Control analyses were rerun without this subject, which did not change the results. This subject was kept to preserve power.

Hippocampal volumes

Our first aim was to identify whether heavy cannabis use had an effect on changes in hippocampal volumes over a three-year period in a sample of young adults. We found a significant main effect of time ($F(1,39)=26.22$, $p<0.001$, $\eta^2=0.40$; Figure 1) indicating that the hippocampus was larger at follow-up relative to baseline. However, we found no significant main effect of group ($F(1,39)=0.17$, $p=0.69$) and no significant group by time interaction effect on hippocampal volumes ($F(1,39)=0.01$, $p=0.93$; Supplementary material, Table 1) indicating that heavy cannabis use did not alter the course of hippocampal volume development in this three-year period. In addition, we found a significant main effect for gender ($F(1,39)=10.33$, $p=0.003$, $\eta^2=0.21$), from which we can infer that males had larger hippocampal volumes than females in both groups, on both time points and in both hemispheres (see supplementary Table 4 and Supplementary Material, Figure 1).

There was a significant interaction effect of time by hemisphere on hippocampal volumes ($F(1,39)=12.04$, $p=0.001$, $\eta^2=0.24$; Supplementary Material, Table 1). The difference between left and right hippocampal volumes was more marked at baseline (mean difference=113.43, $p=<0.001$) than at follow-up (mean difference=76.63, $p=<0.001$) with larger left volumes in all groups and on both time points (Supplementary Material, Table 5). We did not find a significant main effect of age ($F(1,39)=0.001$, $p=0.98$), nor did we find any other (two-way or three-way) interaction effects (Supplementary Material, Table 1). All these effects remained similar when subjects with polysubstance use and/or a comorbid psychiatric disorder were removed from the analysis (Supplementary Material, Tables 7(a) and (b)).

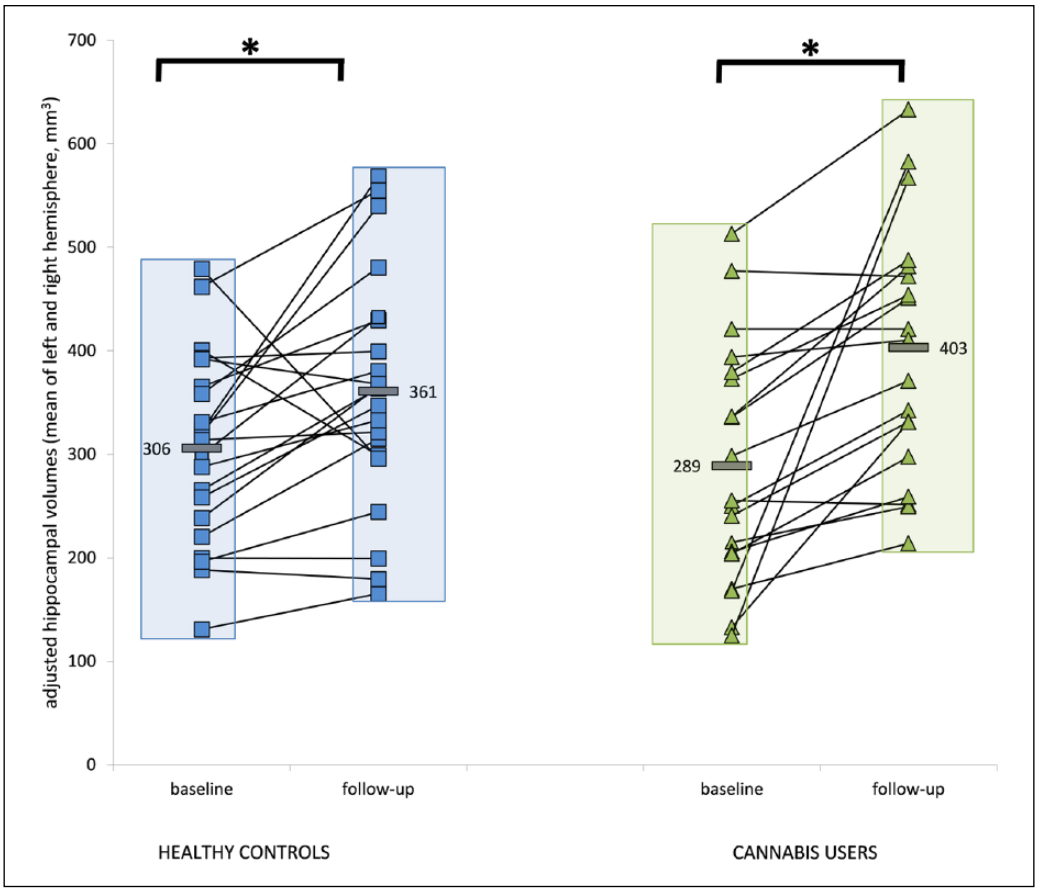

Figure 1. Adjusted hippocampal volumes (mm^3) per group, mean of left and right (blue squares: healthy controls, green triangles: cannabis users) and time point (for each group, left: baseline, right: follow-up). The bars represent mean values for hippocampal volumes. There was a significant effect of time, with larger volumes on follow-up for both groups.

Our second aim was to identify whether cannabis use characteristics (i.e. age of onset of regular use, lifetime cumulative dose and dose change) predicted hippocampal volume changes over time in the group of heavy cannabis users. There were no significant effects of lifetime cumulative dose or dosage change ($F(1,9)=1.94$, $p=0.20$, $\eta^2=0.17$ and $F(1,9)=0.50$, $p=0.50.$, $\eta^2=0.05$ respectively; Supplementary Material, Table 2). However, we did find a trend for an effect of age of onset of regular use on hippocampal volumes ($F(1,9)=3.72$, $p=0.086$, $\eta^2=0.29$; Supplementary Material, Table 2), with a younger age of onset being associated with a larger increase in hippocampal volumes over time (see Supplementary Material, Figure 2). This effect disappeared after excluding the subjects with polysubstance use/and or comorbid psychiatric disorders from the analysis (Supplementary Material, Tables 8(a) and (b)).

Hippocampal shape

We found no group differences, in terms of the longitudinal change on hippocampal shape. We did find a marginal effect of time on the different sub regions of the hippocampus (Supplementary Material, Figure 3); however this did not survive multiple comparison correction. The uncorrected results (of CB users and HCs combined) showed increased volume in the tail or the posterior part of the hippocampus, i.e. near the fornix, and a decrease in the head or the anterior part of the hippocampus, i.e. near the junction with the amygdala. There were no effects of cannabis use characteristics (i.e. age of onset of regular use, lifetime cumulative dosage and dosage change between baseline and follow up) on the changing of hippocampal shape.

Discussion

This is the first longitudinal examination of hippocampal neuroanatomical changes in young adults over a three-year time period. Contrary to our hypothesis, heavy cannabis users showed no significant differences compared to healthy controls in volume nor shape of the hippocampus, neither cross-sectionally, nor over time. Over time, both cannabis users and controls showed overall increases in hippocampal volume, with non-significant enlargements in the tail and shrinkage in the head of the hippocampus. We found no effect of age of onset of regular use, lifetime cumulative dose or dose change on hippocampal neuroanatomical change. These data suggests that cannabis users show the same developmental trends as normative samples and that heavy cannabis use in this group may not necessarily interfere with hippocampal changes in neuroanatomy in early adulthood.

Previous cross sectional studies did find significant differences in hippocampal volume between cannabis users and controls (Ashtari et al., 2011; Demirakca et al., 2011; Lorenzetti et al., 2015; Matochik et al., 2005; Yücel et al., 2008). The combination of these cross-sectional positive findings and the negative findings of our longitudinal study suggests that cannabis-related changes in hippocampal morphometry might already be present in early adolescence but do not worsen after continued heavy use in late adolescence and early adulthood. Moreover, hippocampal differences could predate the initiation of cannabis use and may represent a vulnerability factor for heavy cannabis use or dependence, rather than a consequence of (heavy) cannabis use. This latter interpretation is supported by studies on alcohol-use disorders, which showed that a smaller temporal lobe (which the hippocampus is an integral part of) is a risk factor rather than a consequence of alcohol abuse. For example, smaller temporal lobes have been found in non-addicted adolescents and adults with a positive family history of alcohol dependence (Benegal et al., 2007; Sjoerds et al., 2013). In contrast with this vulnerability interpretation, Cheetham et al. (2012) found that adolescents aged 17–18 years who initiated cannabis use (n=28) showed no pre-existing hippocampal abnormalities, suggesting that structural changes in the hippocampus observed in previous studies are a consequence of chronic, heavy cannabis use rather than a premorbid vulnerability.

Importantly, in line with the present study, many other cross sectional studies found no evidence for smaller hippocampal volumes in cannabis users (Ashtari et al., 2011; Block et al., 2000; Cousijn et al., 2012; Demirakca et al., 2011; Gilman et al., 2014; Lorenzetti et al., 2015; Matochik et al., 2005; Medina et al., 2007; Tzilos et al., 2005; Yücel et al., 2008). Differences in the level of exposure to cannabis may partly explain these inconsistencies. Smaller hippocampal volumes were mostly reported in samples with high levels of cannabis exposure (Lorenzetti et al., 2013). Also Demirakca et al. (2011) reported that hippocampal volume differences between cannabis users and controls depended on the levels of THC relative to cannabidiol (CBD), the main active cannabinoids in cannabis. However, it is unlikely that the cannabis exposure rate in present study was too low to cause changes in hippocampal neuroanatomy as we recruited heavy, long-term cannabis smokers (using on average five days a week, 0.6 g per day for seven years). This is comparable to the amount of cannabis used by the participants in the study of Demirakca et al. (2011), who did report cross-sectional differences in hippocampal volume. Participants in that study smoked on average seven days a week, 0.27 g per day. Also Ashtari et al. (2011) reported cross-sectional differences in a sample that used 3 g per day, but was abstinent for an average of seven months. In addition, heavy cannabis use often co-occurs with a broad range of psychological problems and use of other substances and alcohol. By excluding such participants in the present study, we may have biased the sample towards a more high-functioning group of cannabis users. This may explain our finding and this is supported by higher levels of alcohol use/mental problems in studies that did find a difference

Our results suggest that heavy cannabis use may not interfere with developmental and gender effects on hippocampal neuroanatomy in young adults. Consistent with previous evidence (Lenroot and Giedd, 2010), we found that males had larger hippocampal volumes on both BL and FU assessment. In addition, both cannabis users and controls showed overall hippocampal volume increases, which is consistent with findings in normative samples (Ostby et al., 2009). Regarding the shape analyses, we found non-significant enlargements in the tail and shrinkage in the head. Gogtay (2006) reported similar results in a cross sectional study, with a volume increase in posterior and a decrease in anterior sub regions (Gogtay et al., 2006). Our results concerning hippocampal shape were not statistically significant, which may be due to the stringent multiple comparisons correction and the need to exclude a proportion of data due to failed preprocessing (left hemisphere: 5/43, 11%, right hemisphere: 4/43, 9%). In conclusion, although the present study may have been insufficiently powered to detect subtle changes in hippocampal subregional shape, our findings do point to a gender difference in hippocampal volumes and an increase over time in young adults.

Regarding age of onset we found that an earlier age of onset was (non-significant, p=0.086) associated with more marked increase of hippocampal volumes over time. This trend effect was independent of cumulative dose and change in dose. When taking the normal developmental trajectory into account (with hippocampal volumes increasing until late adulthood (Goddings et al., 2014; Ostby et al., 2009), and subsequently slightly plateauing or decreasing (Ostby et al., 2009) a larger volume increase in the early onset cannabis users could indicate a 'delayed' developmental trajectory, i.e. hippocampal volumes of cannabis users starting at a younger age increase more during young adulthood. Although in need of empirical testing, this could mean that hippocampal volumes might reach a normative volume over time, although delayed. This delay in development could be an explanation for the adverse effects that are found in early onset cannabis users, like early school leaving (Lynskey et al., 2003) and poorer cognitive function (Gruber et al., 2012). This may indicate an adverse effect of cannabis use at an early age on the hippocampus or an association between hippocampal volume and the vulnerability for early start of cannabis use. However, due to the relatively small numbers the present study might have been underpowered to detect smaller hippocampal volume in association with earlier cannabis use. Future longitudinal studies in larger samples of regular cannabis users should further evaluate the role of early onset cannabis use on the developing hippocampus.

Strengths and limitations

For the first time, this study examined whether continued cannabis use has an effect on changes in hippocampal neuroanatomy over time, using a within-subject design. The detailed measurement of cannabis variables (age of onset of regular use, lifetime cumulative dose or dose change) in the examined cannabis group allowed us to study the influence of potential mediating variables, including cannabis dose and age of onset. Last, the use of manual tracing has the benefit of enhanced sensitivity to subtle effects in hippocampal shape and greater precision in detecting inter-individual variability in anatomical boundaries when compared to the application of automated protocols (Stjepanovic et al., 2013). Manual tracing also allows shape analyses, enabling the examination of whether cannabis use affects specific hippocampal subregions (Gilman et al., 2014).

This study however also has some limitations. First, confounding factors that also affect brain neuroanatomy (psychopathology

and comorbid substance use) may have affected our findings. Cannabis users were diagnosed more often with psychiatric disorders (depressive disorders, *n*=3 and ADHD, *n*=3). In addition, in comparison with controls, cannabis users used on average a higher amount of cigarettes and other drugs. We controlled for these potential confounding effects by repeating the analyses with and without individuals with comorbid psychiatric disorders, and with higher levels of use of substances other than cannabis. This did not influence our pattern of results. Alcohol use was matched between the groups, and did not affect our findings. Second, our sample did not contain subjects that were cannabis-naïve at the start of the study and then started to use cannabis persistently for at least three years. Therefore we cannot rule out the possibilities that hippocampal volumes were affected during the early course of cannabis use or the change in volumes was related to factors influencing the onset of cannabis use. Future studies such as the prospective Adolescent Brain Cognitive Development (ABCD; NIMH, USA) study will hopefully be able to distinguish if temporal lobe deficits represent a vulnerability factor for or a consequence of heavy cannabis use.

In conclusion, our current findings confirm our previous VBM findings in the same cohort (Koenders et al., 2016), suggesting that cannabis use does not affect hippocampal neuroanatomical changes in early adulthood, since cannabis users showed similar developmental trends as normative samples. However, since other studies have suggested detrimental effects of (heavy) cannabis use on both cognitive and other MRI measures, there is a need for future longitudinal studies in larger samples of regular cannabis users to confirm the role of early onset cannabis use on the developing brain.

Acknowledgements

The following author contributions were made: LK, JC and WAMV were responsible for the recruitment of patients and healthy control participants and the collection of data. VL provided supervision and training of LK for the manual tracing and statistical analyses of the hippocampi. CS assisted with the shape analyses. LK was responsible for analysing the results and drafting of the manuscript. JC, WvdB and MWJM assisted with interpretation of findings. WvdB, RWW, CJM, MWJM, DJV, AEG, MY and LdH provided critical revision of the manuscript. All authors critically reviewed content and approved the final version for publication.

Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study is supported through funding received from the Academic Medical Center of the University of Amsterdam and the Netherlands Organization for Scientific research – Health Research and Development, ZonMW grant #31180002 awarded to AE Goudriaan and W van den Brink and grant #31160007 awarded to L de Haan. Additional funding was obtained from Vici grant #453.008.001 awarded to RW Wiers by the Dutch Organization for Scientific Research (NWO) and from the Amsterdam Brain Imaging Center (BIC) for MRI scans. M Yucel was supported by a National Health and Medical Research Council Fellowship (#1117188) and the David Winston Turner Endowment Fund.

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