Daily Caffeine Intake Induces Concentration-Dependent Medial Temporal Plasticity in Humans:

A multimodal double-blind randomized-controlled trial

Yu-Shiuan Lin[1,2,3], Janine Weibel[1,2], Hans-Peter Landolt[4,5], Francesco Santini[6,7], Martin Meyer[1,2,8], Julia Brunmair[9], Samuel M. Meier-Menches[9], Christopher Gerner[9,10], Stefan Borgwardt[3], Christian Cajochen[1,2*], Carolin Reichert[1,2]

1 Centre for Chronobiology, Psychiatric Hospital of the University of Basel, Switzerland
2 Transfaculty Research Platform Molecular and Cognitive Neurosciences, University of Basel, Switzerland
3 Neuropsychiatry and Brain Imaging, Psychiatric Hospital of the University of Basel, Switzerland
4 Institute of Pharmacology and Toxicology, University of Zurich, Switzerland
5 Sleep & Health Zurich, University Center of Competence, University of Zurich, Switzerland
6 Department of Radiology, Division of Radiological Physics, University Hospital Basel, Switzerland
7 Department of Biomedical Engineering, University of Basel, Switzerland
8 Clinical Sleep Laboratory, Psychiatric Hospital of the University of Basel, Switzerland
9 Department of Analytical Chemistry, University of Vienna, Austria
10 Joint Metabolome Facility, University of Vienna and Medical University of Vienna, Vienna, Austria

Address for correspondence:
Prof. Christian Cajochen, PhD
Centre for Chronobiology
Psychiatric Hospital of the University of Basel
Wilhelm-Klein Strasse 27
CH-4002 Basel
Tel: +41 61 325 53 18
Fax: +41 61 325 55 56
Email: Christian.cajochen@upkbs.ch

Introduction

Caffeine is the most commonly used psychostimulant worldwide and mainly consumed in forms of coffee, tea, energy drink, and soda (Barone and Roberts 1996; Frary and others 2005; Mitchell and others 2014; Reyes and Cornelis 2018). Although caffeine is mostly considered to be non-addictive, the observed physical and psychological dependence (Mills and others 2016; Nehlig 1999) consolidate its regular consumption (Ferre 2008; 2016; Fredholm and others 1999) through the caffeine-induced reinforcing effects (Griffiths and Woodson 1988), as well as the motive to resist withdrawal symptoms (Juliano and Griffiths 2004) and to increase alertness (Mahoney and others 2018). Higher alertness after acute caffeine intake (Einother and Giesbrecht 2013) mirrors a reduced homeostatic sleep pressure, which is also evident in a reduced depth of sleep (Clark and Landolt 2017). This is characterized by attenuated electroencephalographic slow-wave activity (EEG SWA, 0.75 – 4.5 Hz) in non-rapid eye movement (NREM) sleep and shortened slow-wave sleep (SWS) (Clark and Landolt 2017; Drapeau and others 2006; Landolt and others 1995a; Urry and Landolt 2015).

Disturbed sleep homeostasis can cause not only cerebral micromorphometric alterations in the mitochondria and chromatin that leads to cell death (Abushov 2010; Zhao and others 2016; Zhao and others 2017), but also macrostructural changes. Lower grey matter (GM) volumes were observed during abnormally high sleep pressure, such as during sleep deprivation or sleep fragmentation. Liu and others (2014) reported reduced thalamic GM volume along with impaired cognitive performance in healthy adults after 72-hour prolonged waking compared to baseline. Dai and others (2018) demonstrated a GM dynamic through the 36-hour course of sleep deprivation, where a decrease in right thalamus, right insula, right inferior parietal lobe, and bilateral somatosensory association cortex was observed by 32 hours of sleep deprivation. In clinical studies, GM volume and cortical thickness were reduced in patients with various sleep disorders (e.g. chronic insomnia (Altena and others 2010; Joo and others 2013), sleep apnea (Baril and others 2017), and narcolepsy (Joo and others 2011)) compared to healthy controls. Together, the micro- and macro-morphometric changes in GM in response to sleep deprivation might reflect a disrupted adenosine-

modulated cellular homeostasis, such as cardiac microtubule dynamic (Fassett and others 2009), astrocytic cytoskeleton arrangement (Abbracchio and others 2001), hippocampal fiber synaptic plasticity (Kukley and others 2005), and the robustness of cortical axons and dendrites (Ribeiro and others 2016; Ribeiro and Sebastiao 2016).

Caffeine has been shown in animals to exert neuroprotective effects through the A2A receptor antagonism on age-related (Prediger and others 2005), disease-related (Laurent and others 2014), stress-associated (Kaster and others 2015), or kainate-induced (Cognato and others 2010) cognitive decline. However, caffeine also interferes with sleep homeostasis through antagonizing A1 and A2A receptors (Elmenhorst and others 2012; Urry and Landolt 2015). Despite of its popular use, it remains unclear whether daily caffeine consumption in humans has long-term impact on cerebral structures through the constant impact on sleep homeostasis.

Hence, we hypothesized that, through the impacts on sleep homeostasis, daily caffeine intake alters GM structures. We measured cerebral GM volume by magnetic resonance imaging (MRI) after 10 days of standardized caffeine intake (vs placebo) in young healthy habitual caffeine consumers during a strictly controlled laboratory protocol. To control for biases on the MRI signals, we adjusted the GM responses for caffeine-induced changes in cerebral blood flow voxel-to-voxel. As an objective measure to indicate homeostatic sleep pressure, we focus on sleep SWA (0.75- 4.5 Hz) derived from frontal regions in the first non-rapid eye movement (NREM) sleep episode (i.e. from sleep stages except REM within the first sleep cycle). This frequency band is known to be most sensitive toward the variations in sleep pressure and the effect of caffeine (Carrier and others 2009; Dijk and others 1997; Landolt and others 1995a; Werth and others 1997).

Methods

The study was approved by the Ethics Committee northwest/central Switzerland (EKNZ 2016-00376). The study execution followed the declaration of Helsinki, and all participants were fully informed with study details and consented in written form.

Recruitment and Participants

Applicants aged between 18 and 35 years, BMI ≥ 18 and ≤ 25, non-shift workers, and without a history of transmeridian travels <1 month prior to study, were screened according to the following exclusion criteria:

- self-reported caffeine intake <300 or >600 mg/day (calculations were based on the (Bühler and others 2013), adapted according to a classification of (Snel and Lorist 2011) to ensure the safety of caffeine intake and to exclude extreme response
- bad sleep quality, i.e. PSQI> 5 in the last four weeks assessed by the Pittsburg Sleep Quality Index (PSQI) to control for sleep disturbances
- extreme chronotype, as defined by Horne-Ostberg's Morningness-Eveningness Score (HOMES) ≤ 30 or ≥ 70 to prevent pronounced variance in circadian phase.
- self-reported regular substance use (including medication, nicotine, and drugs) and other major medical conditions.

A habituation night in the laboratory was conducted to exclude poor sleep efficiency (SE < 70%) and clinical sleep disturbances (apnea index > 10, periodic leg movements > 15/h). A toxicological screening right before each laboratory session served to exclude the influence of recent drug intake including cannabis, amphetamine, methamphetamine, cocaine, benzocaine and morphine.

Participants, Study Protocol and Environmental Control

Overall, 20 healthy male participants completed the study. Here, we focus on the within-subject comparison of a 10-day caffeine and a 10-day placebo condition in randomized order (10 participants in the order of caffeine – placebo and 10 in placebo – caffeine, the conditions were apart minimal 11 days and no longer

than 2 months). The average age was 26.4 ± 4.0 years, body mass index (BMI) 22.7 ± 1.38 kg/m2, and self-reported daily caffeine intake was 474.1 ± 107.5 mg/day.

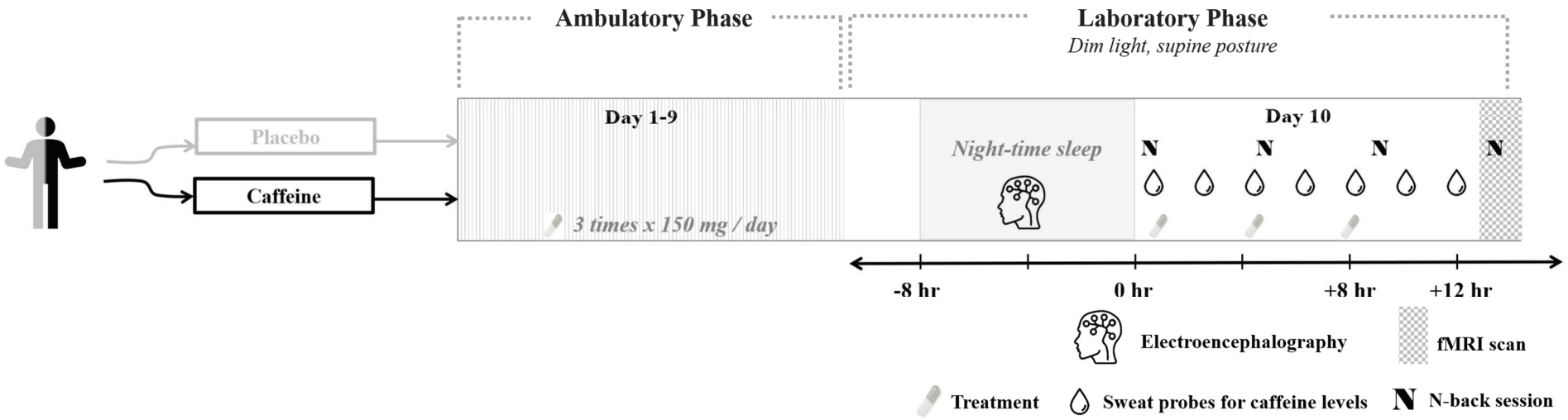
Each protocol consisted of 9 days of ambulatory phase, followed by the strictly controlled laboratory stay (**Fig 1**). In order to examine the effects from daily caffeine intake and to avoid withdrawal effects during the laboratory phase at abstinence (Juliano and Griffiths 2004), the treatment, caffeine (3 x 150 mg/day) or placebo (mannitol) capsules, had been administered for 9 days prior to the laboratory phase. Timing of intake was set to 45 minutes, 4 hours, and 8 hours daily after waking up to imitate the pattern of caffeine intake in reality (Martyn and others 2018). During the 9 days of ambulatory phase, participants complied to a fixed sleep-wake cycle (8 hours ± 30 minutes in bed, no naps allowed) to control for high sleep debt. Individual bedtimes were chosen according to usual bedtimes of each participant (Tinguely and others 2014). The compliance to the sleep schedule was monitored by actimetry and sleep diaries. Furthermore, participants were asked to abstain from caffeine-containing diets including coffee, tea, energy drink, soda, and chocolate, etc. To check for compliance to the treatments, participants were instructed to collect fingertip sweat samples during the ambulatory phase with standard cleaning procedure every evening 2 hours before bedtime. Sweat from the fingertips is an emerging tool for metabolomic biomonitoring in humans (Brunmair and others 2020).

In the evening of the 9th day, participants started the laboratory phase, where they stayed in dim light (< 8 lux), constant half-supine position (~45°), with controlled dietary and lavatory time. Water consumption was allowed ad libitum and did not differ between conditions (see **Figure S3** in supplementary materials for more information). Access to mobile or other forms of social contacts was forbidden. The participants slept at their habitual bedtime with polysomnographic recording.

On the 10th day after waking up, the treatment (caffeine vs placebo) continued at identical times as during the ambulatory phase (see **Fig 1**). The time of the protocol was adapted to individual's habitual bedtime. A comparison of salivary dim-light melatonin onset (DLMO) did not indicate a significant difference in circadian timing between caffeine and placebo (see Figure S4 for the course of salivary melatonin per condition). More comprehensive results of circadian-associated variables were reported in Weibel and

others (2020b). MRI measurement was scheduled to start at 12.75 hours after awakening (roughly 13.5 hours for ASL sequence, equivalent to 5.5 hours after last caffeine treatment). Visual working memory tasks (N-back) and measurements to assess caffeine levels and caffeine metabolites in the sweat of the fingertip were operated every 4 hours and every 2 hours, respectively.

Figure 1. Overview of the study design. Every participant underwent both a placebo and a caffeine condition. Each condition consisted of 10 days – 9 days of ambulatory phase with treatment, followed by the night-time sleep and the laboratory day. On the 10th day, the MRI scan started at 12.75 hours of EEG-monitored wakefulness (roughly 13.5 hours for ASL sequence, equivalent to 5.5 hours after last caffeine treatment). Levels of caffeine and paraxanthine were measured in the fingertip sweat every 2 hours, and visual working memory tasks (N-back) were performed every 4 hours through the laboratory phase.



Data acquisition

1. MRI

T1-weighted structural data were obtained with a three-dimension (3D) magnetization-prepared rapid gradient-echo (MP-RAGE) sequence (1x1x1mm^3, TR=2000ms, TI= 1000ms, TE=3.37ms, FA=8°) on a 3T Siemens scanner (Prisma; Siemens AG, Erlangen, Germany). Cerebral blood flow (CBF) was measured by 2D-echo-planar imaging pulsed arterial spin labeling (ASL) sequence (4x4x4mm^3, TR=3000ms, TE=12ms, FA=90°, FoV=100) at the same MR scanner.

2. Sleep EEG

Polysomnography was operated via V-Amp devices (Brain Products GmbH, Gilching, Germany) with a sampling rate of 500 Hz and notch filter at 50 Hz. The recording was conducted on the identical device between conditions of each subject. Electrophysiological activity was recorded above frontal (F3, F4), central (C3, C4), and occipital (O1, O2) regions against the linked mastoids (A1, A2) as the reference electrodes, as well as electro-oculography, electro-myography, and electro-cardiography to determine sleep stages. A thorough investigation of sleep and circadian variables has been reported elsewhere (Weibel and others 2020a).

3. Caffeine and metabolites from fingertip sweat

Considering the inter-individual variance in caffeine metabolism (Nehlig 2018), we measured the individual levels of caffeine and caffeine metabolites in the sweat from the fingertips during the ambulatory and laboratory phases. Samples were collected once daily during the ambulant phase, while the laboratory phase included 7 times repeated sampling in 2-hour intervals from the awakening until the MRI scan. Sweat was collected using 1 cm^2 sampling unit. Samples were then processed and analyzed according to Brunmair and others (2020). In short, the metabolites were extracted from the sampling unit using an acidified aqueous solution and subjected to analysis by liquid chromatography-mass spectrometry (LC-MS) using a Q Exactive HF orbitrap hyphenated with a Vanquish UHPLC

chromatography system (both ThermoFisher Scientific). The collected samples were shipped from Basel (CH) to Vienna (AT) by postal service, where they were processed.

4. N-back task

Performance in visual working memory N-back tasks was exploratorily analyzed based on the results in GM in order to test whether a concomitant change in the memory domain was present. Four sessions were completed in 4-hour intervals during the 13 hours of wakefulness and lasted approximately 15 minutes per session. Every session consisted of 9 trials of 3-back (30 stimuli each) and 5 trials of 0-back (30 stimuli) in quasi-random order. During each session, a series of letters was presented on the computer screen. The participants were asked to press key "1" when the letter presented was the same as three stimuli before (3-back condition) or was a "K" (0-back condition), while pressing "2" when it was not. For habituation, participants performed one practice session on the 9th day evening. The differences between the caffeine and placebo conditions in the average accuracy and reaction time (RT) of four sessions are reported.

Data Preprocessing and Analyses

The pipeline of the imaging analyses in the present study consisted of three steps: a) Determining the condition effects in GM by whole-brain voxel-based analysis, b) adjusting the GM results for the inter-interpersonal variance of CBF voxel-to-voxel, and finally, c) extracting the first eigenvariates of the cluster exhibiting significant GM differences for further analyses with other covariates, i.e. SWA, areas under the curves of the caffeine/paraxanthine levels (AUC-CAPX), and working memory performance.

1. MRI data for GM and CBF

For the preprocessing of T1-weighted images, we used CAT12, an extension toolbox of SPM12 (University College London, London, UK). We adopted the built-in pipeline for repeated measures, where volumetric measures were co-registered to the mean of the two volumes from each participant. Total intracranial volume (TIV) and the volumes of each cerebral compartment (grey matter, white

matter, and cerebrospinal fluid) of each participant were segmented based on an affine registration on a tissue probability map in SPM12. The modulated normalization was carried out by registering each participant's image collected in the placebo condition as a baseline onto an MNI-defined standard brain (Montreal Neurological Institute, McGill University) and applying the estimation onto the image collected in caffeine condition. A Gaussian kernel of FWHM= 8 x 8 x 8 mm^3 was adopted for the smoothing process.

ASL images were preprocessed with FMRIB Software Library (FSL) 5.0 developed by the Oxford Center for Functional MRI of the Brain (United Kingdom). Motion correction was estimated, followed by the generation of M0 calibration volume and tag-control pairs to calculate relative and quantify absolute CBF maps. The final absolute CBF maps were co-registered onto the individual T1-weighted images and MNI space.

For the first step of the pipeline (a), the condition effect in *total* GM volumes, which were estimated during the segmentation process, were tested by generalized linear mixed model (Gamma distribution) in R (R core team, Vienna, Austria). To specify *regional* differences of the condition effects (caffeine vs placebo), on GM and CBF, we used a flexible factorial model in SPM12 and pair-T (equivalent to linear mixed model) on FSL, respectively. Nonparametric threshold-free cluster enhancement (number of permutations = 5000, cluster-level threshold $p < .01$) was further performed by the SPM TFCE toolbox and FSL "*randomise*" function on GM and CBF, respectively. A mask of GM generated from the template was applied to reduce the bias from the correction of multiple comparisons.

Since caffeine-induced reductions in CBF can bias the MRI signal distribution (Field and others 2003; Ge and others 2017; Laurienti and others 2003), for the second step (b), the VoxelStats toolbox (Mathotaarachchi and others 2016) was applied to, voxel-to-voxel, control for the covariance of CBF and GM when estimating the coefficient of condition effects in a linear mixed model (cluster-level threshold $p_{FDR} < .001$).

To relate (c) condition-specific regional differences in GM to sleep, caffeine levels, and working memory performance (as specified below), we extracted the first eigenvariates of GM in the cluster exhibiting a significant change from the whole-brain analysis. We examined the associations with other variables using a linear mixed model in R (R core team, Vienna, Austria).

All the GM models were adjusted for individual total intracranial volumes (TIV).

2. EEG slow wave activity

To determine NREM, two treatment-blinded experimenters (Y.-S. L., and J. W., inter-rater reliability above 85%) visually scored all nights in 30s epoch based on AASM (Berry and others 2012). Artefacts were detected visually and manually rejected during the scoring. The power density of SWA during NREM sleep was quantified separately for each 0.25 Hz bin in the frequency range of 0.75 – 4.5 Hz by a Fast-Fourier-Transform spectrum analysis of the signal recorded over frontal electrodes (4s spectrums over 30s epochs averaged, window function = hamming, 0% overlapped). Results per condition and bin are illustrated in supplement (**Fig S1**). The condition effect (caffeine vs placebo) in EEG SWA was analyzed through generalized linear mixed model on R. We used a gamma distribution model to estimate the raw EEG data instead of using Gaussian on log-transformed data to maintain the originality of the data interpretation, as the data in log-transformed scale yield a slightly different meaning from the original scale (Ng and Cribbie 2017). In addition, we determined the best-fit model between two methods by the lower Akaike information criterion and Bayesian information criterion, as well as visual examination of Q-Q plots of residuals.

3. Caffeine and paraxanthine levels

We kept all values original including the ones below limit of detection. To estimate the individual amount of caffeine exposure, we combined the area under the curves of caffeine (CA) and paraxanthine (PX), abbreviated CAPX, as paraxanthine also antagonizes adenosine at A_1, A_{2A}, and A_{2B} receptors (Cornelis and others 2016), while it follows a similar but slower metabolic pattern as compared to caffeine. To investigate the association of acute caffeine and paraxanthine exposure with other variables

(such as GM or SWA), the area under the curve was calculated with the trapezoidal rule over the 7 samples from the awakening until the scan. The dynamic of AUC-CAPX level over the 12.75 hours was illustrated in the supplement (**Fig S2**).

4. N-Back

In the n-back tasks, the "correct rate" was defined as (hits + correct rejection)/all responses + no response, while the "incorrect rate" was (missed + false alarm)/all responses + no response. Accuracy was defined as the ratio of correct rate to incorrect rate. The mean reaction time of overall as well as of each type of responses was calculated over 9 trials of 3-back and over 5 trials of 0-back, respectively, in each session. The condition effect (caffeine vs placebo) in the performance of N-back tasks was analyzed through generalized linear mixed model (Gamma distribution) on R. The orders of the conditions were adjusted in order to reduce the impact of learning effect between conditions.

Results

Caffeine induces a decrease in grey matter (GM) volume

Sweat from the fingertip was used to monitor compliance during the ambulatory and laboratory phases. During the laboratory day until the MRI scan we found significantly higher levels in the caffeine than in the placebo condition by combining the area under the curves of caffeine and paraxanthine levels (AUC-CAPX, Fig S2, $t = 13.60$; $p < .001$; 95% CI of coefficients = [2.473, 3.305]; $R^2m = 0.70$; $R^2c = 0.85$).

Total GM volume was lower in the caffeine condition compared to placebo ($t = -3.59$; $p < .001$; 95% CI of coefficients = [-0.009, -0.003]; $R^2m = 0.75$; $R^2c = 0.97$, **Fig 2**). A voxel-based analysis (VBA) indicated that the reduction of GM volume was most prominent in the right medial temporal lobe (*mTL*, including hippocampus; VBA threshold: cluster level $p_{FWE} < .05$, **Fig 3**), along with the following regions at trend (i.e. $0.05 \leq p_{FWE} < .063$) of a reduction: left frontal pole, right postcentral gyrus, right insula, and the cerebellum. Both *total* GM and *mTL* GM (**Fig 4**) were negatively associated with AUC-CAPX, i.e. the larger the reduction was in GM the higher was the individual AUC-CAPX (statistics see **Table 1**). No significant increases of GM in the caffeine compared to the placebo condition were observed.

Figure 2. The associations of *total* grey matter volume with *total* cerebral blood flow and caffeine metabolites. We use center plots to display the changes in a specific variable to the treatment of caffeine and placebo in each participant. The values are the relative distance from the responses in each condition to the average response of a single participant, calculated as $response_{caff\ or\ plac} - (response_{caff} + response_{plac})/2$. Each symbol represents one participant. In two dimensions one can observe the variance of within-subject changes of two variables between conditions (by color), as well as observe the association between the changes of two variables (x and y axes). The stronger the condition effect is, the more discretely are the two-colored clouds distributed. The stronger the association between two variables is, the closer the shape is to linear. (a) The lower *total* grey matter volume was associated with higher AUC-CAPX. (b) The lower *total* grey matter volume was associated with lower *total* cerebral blood flow.

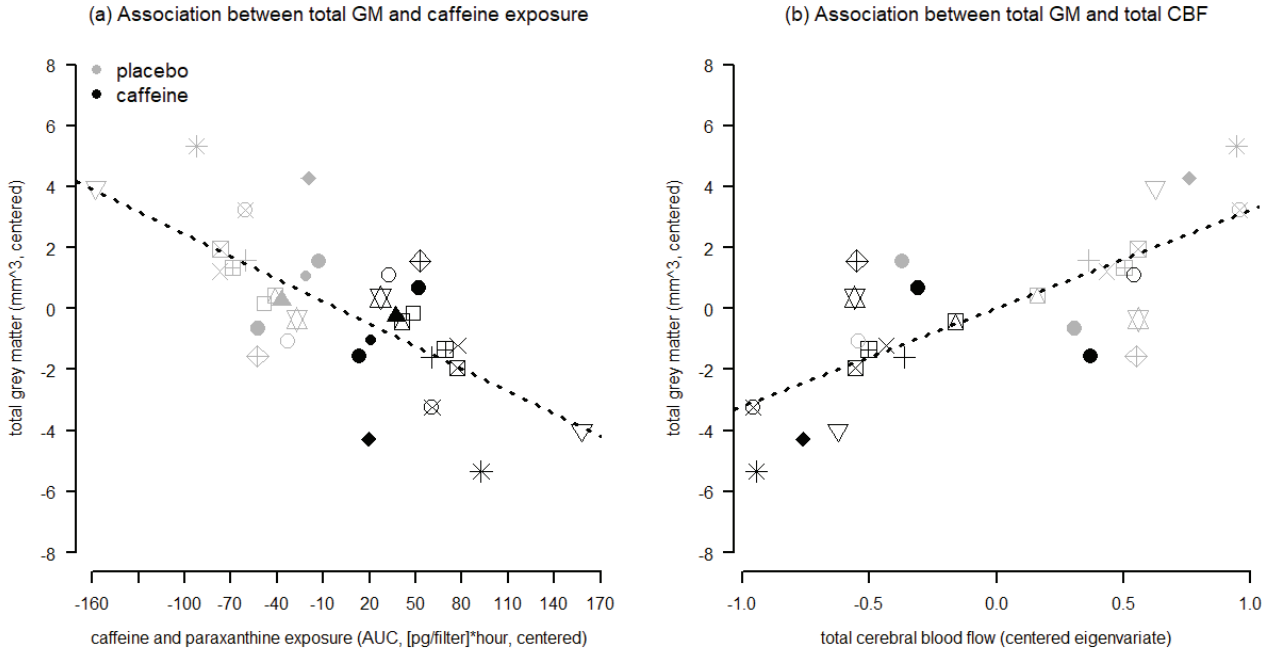


Figure 3. Reduction in medial temporal grey matter. The blue area indicated the clusters that showed a significant GM reduction in medial temporal lobe (at combined voxel-cluster-level $p_{uncorrected} < 0.001$, $p_{FWE} = .032$) in the caffeine condition compared to placebo, based on a voxel-based non-parametric analysis.

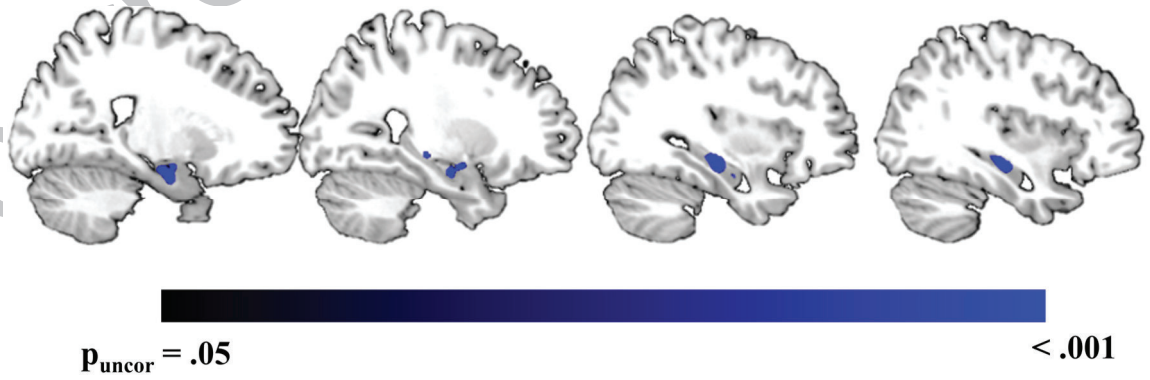


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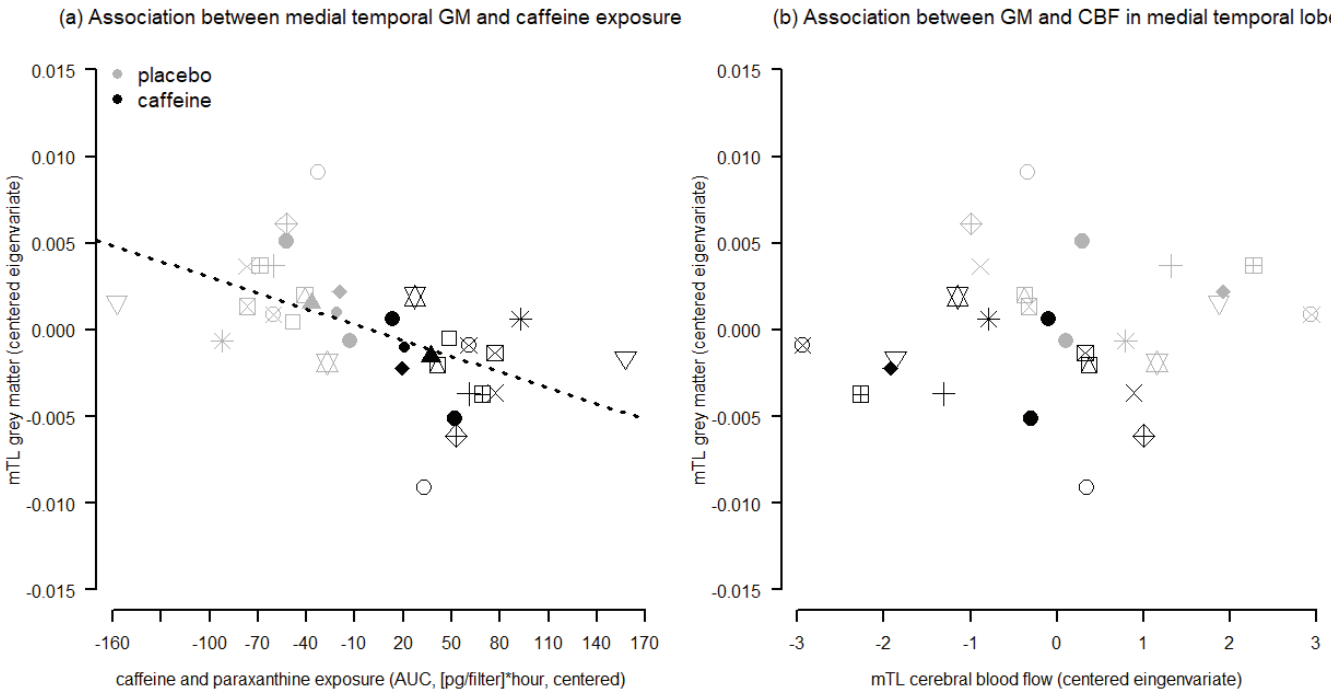
Figure 4. The associations of medial temporal grey matter volumes with medial temporal (*mTL*) cerebral blood flow and caffeine metabolites.

(a) The individual response in grey matter volume within the significant *mTL* clusters was positively associated with the levels of caffeine and paraxanthine. (b) No significant association between *mTL* grey matter volumes and *mTL* cerebral blood flow was found.



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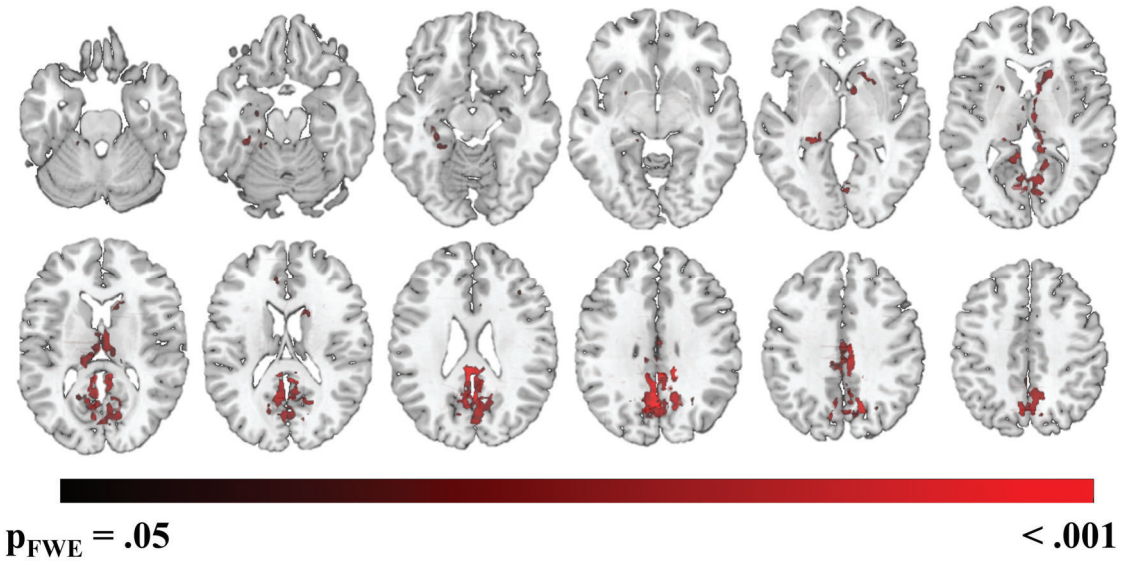
Cerebral blood flow (CBF) remains reduced at 5.5 hours after the last caffeine intake

Compared to the placebo condition, *Total* CBF was significantly reduced in the caffeine condition ($t = -5.2$; $p < .001$; 95% CI of coefficients = [-0.119, -0.054]; $R^2m = 0.12$; $R^2c = 0.71$). Voxel-wise analysis suggested that the reductions occurred prominently in the midline, including cuneus, precuneus, and subcortical regions (cluster level $p_{FWE} < .05$) (**Fig 5**). AUC-CAPX was negatively associated with both *total* CBF and *mTL* CBF (statistics see **Table 1**).

Figure 5. Regional differences in cerebral blood flow. Regions in red indicate the reduced cerebral blood flow in cuneus, precuneus, and subcortical regions ($p_{FWE} < .05$) after caffeine intake compared to placebo.



Cerebral blood flow (CBF) changes account for caffeine-associated total but not regional grey matter (GM) differences

Examining the inter-individual variances between the changes in GM and CBF, only the *total* CBF and *total* GM were positively associated, yet no association between *mTL* CBF and *mTL* GM was observed (**Table 1**). By extension, *total* CBF predominantly accounted for the variance of *total* GM and divested the explanatory power of the condition effect on *total* GM in the two-factor model, indicating a mediation effect

of CBF on the observed changes in *total* GM (**Fig 2**). On the contrary, *mTL* CBF did not account for the reductions in *mTL* GM, determined by both the multi-modal voxel-based and ROI-based linear mixed models (**Fig 4**). The detailed statistics of the effects of covariates are presented in **Table 1**.

No significant association of sleep SWA caffeine-induced regional GM changes

In NREM SWA we did not observe significant differences between caffeine and placebo conditions (t = -8.87, p = .0386). However, NREM SWA was positively associated with *total* GM, and a significant interaction between condition and SWA indicated a stronger association in the placebo condition. No significant associations between SWA and *mTL* GM volumetric reductions were seen **(Table 1)**.

Exploratory analyses indicate poorer working memory performance during daily caffeine intake

As an exploratory step to observe whether a change in the memory domain might be present concomitantly with the *mTL* GM changes, we tentatively inspected into the only memory-related assessment (N-back) conducted in the study. The inter-individual variance of the reductions in response accuracy was not associated with the magnitude of *total* nor *mTL* GM reduction, however, a relatively poorer working memory performance was found in caffeine condition: Compared to placebo, lower accuracy was found in both 0-back (t = -2.33, p = .020) and 3-back (t = -2.02, p = .044) performance in the caffeine condition, as well as a lower *net* accuracy, i.e. response accuracy in 3-back corrected for the baseline response in 0-back performance (t_{con} = -1.97, p_{con} = .049; R^2m = .41, R^2c = .85**)**. Furthermore, the RT of missed (t_{con} = 2.75, p_{con} = .006; R^2m = 0.87; R^2c = 0.99) and correction rejections (t_{con} =1.95, p_{con} = .051; R^2m = 0.90; R^2c = 0.99) were longer in caffeine condition compared to placebo, albeit no difference in the overall and in hits and false alarm responses (t_{all} < 1.88, p_{all} > .60). Within the caffeine condition, a higher AUC-CAPX level was associated with a better *net* accuracy (t_{AUC} = 3.58, p_{AUC} < .001; R^2m = 0.87; R^2c = 0.99) and a shorter *net* RT in all types of responses (t_{all} < -2.44, p_{all} < .015; R^2m_{all} = 0.87; R^2c_{all} = 0.99).

Table 1. The effect of treatment and pairwise associations between all physiological variables. The information given in each line of each cell corresponds to: Line (1) the direction of the association, (2) t and p value, (3) 95% confidence interval of the coefficients (noted in [lower limit, upper limit]), and (4) the effect size of the coefficients (noted in [R^2margianl, R^2conditional]). The R^2 marginal includes only the variance of the fixed factors, while R^2 condition includes the variance of all (fixed + random factors).

		Dependent variables				
		Total GM	*mTL* GM	*Total* CBF	*mTL* CBF	SWA
Independent variables	AUC-CAPX	Negative t = -3.5; p = .001 [-0.00008, -0.00002] [0.76; 0.98]	Negative t = -3.8; p = .001 [-0.004, -0.001] [0.42; > 0.99]	Negative t = -6.3; p = .001 [-0.0009, -0.0005] [0.26; 0.75]	Negative t = -2.8; p_{AUC} = .055 [-0.021, -0.004] [0.32; 0.63]	N.S. t = -0.8; p = 0.441 [-0.002; 0.001] [0.01; 0.74]
	Total CBF	Positive ∓ t = 4.70; p < .001 [0.004, 0.009] [0.74; 0.98]	--	--	--	--
	mTL CBF	--	N.S. t = -1.7, p = 0.122 [-0.003, 0.0002] [0.51; 0.99]	--	--	--
	SWA	Positive* t = 2.9; p = .004 [0.00008, 0.0004] [0.78; 0.98]	N.S. t = 0.1, p = .931 [-0.0004, 0.0005] [0.57; > 0.99]	N.S. t = 0.2; p = 0.861 [-0.002; 0.002] [0.03; 0.72]	N.S. t = -0.1; p = 0.928 [-0.003; 0.003] [0.11; 0.57]	--

N.S. indicates that the p value is below threshold (> .05), or when 95% confidence interval of coefficients covers 0.

* Statistically significant interaction ($t_{c\text{-}p}$ = -4.709, p < .001): This positive association between SWA and *total* GM was significantly attenuated in the caffeine condition compared to in placebo.

∓ Including *total* CBF as a covariate fully accounted for the main effect of condition on *total* GM estimation (t_{CBF} = 2.82, p_{CBF} = .005; t_{con} = -0.77, p_{con} = .441).

Discussion

Our study examined whether daily caffeine intake affects human grey matter (GM) through the mediation of homeostatic sleep pressure, indexed by sleep EEG slow-wave activity (SWA). We observed a GM reduction in *total* volume, which was however artificially confounded by changes in cerebral blood flow (CBF), suggesting to strictly control for caffeine intake in future MRI morphometric studies. Irrespective of CBF adjustment, a caffeine-induced concentration-dependent reduction in GM volume was present specifically in a cluster within the medial temporal lobe (including hippocampus, parahippocampus, fusiform gyrus). In contrast to our expectations, night-time sleep SWA did not explain these reductions. In other words, GM plasticity, particularly in the medial temporal region, might be induced by daily caffeine intake through a parallel pathway to its acute influence on sleep. Together with our observation on the poorer working memory performance, our data represent an important piece of knowledge regarding the impact of the most common psychostimulant on human brain structure and performance. We strongly indicate to adopt a fine-grained perspective on the divergent effects of caffeine after acute intake in comparison to daily use.

Based on the evidence of sleep-homeostatic effects on brain morphology (Dai and others 2018; Liu and others 2014), we assumed that daily caffeine intake may consequently affect GM volumes. While the data indeed support the latter, this observed alteration did not result from a proportionate disturbance in homeostatic sleep pressure during nighttime and thus may suggest another mechanism. While caffeine reinstates the adenosine-inhibited synaptic excitatory signaling in human neurons (Kerkhofs and others 2017) and attenuates synaptic long-term potentials (Costenla and others 2010; Lopes and others 2019), SWA during sleep mirrors the renormalization of synaptic capacity and recovery of brain neurons from the energy consumption during prior wakefulness (Tononi and Cirelli 2003; 2014). We speculate that, between the energy usage during wakefulness and the recovery of neurons at sleep, daily caffeine intake perhaps did not slant the balance by compromising the side of recovery process but raising the side of energy usage. In other words, an enhanced excitation in neurons induced by caffeine might increase the *need* of synaptic

recovery, which was, however, incompletely fulfilled in a regular sleep and even further regained by the next-day consumption. The *mTL* GM plasticity observed in the present study might be a consequence of the lack of commensurate synaptic restoration from the cellular stress in a long run.

A few reasons might account for the co-occurrence of a caffeine-induced response in CBF and the absence of any clear-cut changes in SWA. First of all, there was a difference in timing of measurements. CBF was measured at 5.5 hours after the last intake, while the start of nighttime sleep was set to 8 hours after the last intake. The previous evidence with different durations from the last intake suggests that a longer duration may attenuate the effect of caffeine on sleep (Drapeau and others 2006; Landolt and others 1995a; Landolt and others 1995b). Moreover, different subtypes of adenosine receptors (A_1R and $A_{2A}R$) may mediate caffeine-induced changes in CBF and SWA. A_1R has much stronger propensity to develop a tolerance to daily caffeine treatment compared to $A_{2A}R$ (Halldner and others 2000; Johansson and others 1993; Johansson and others 1997; Karcz-Kubicha and others 2003; Popoli and others 2000). Thus, daily caffeine intake remains its effect in CBF through the blockade of $A_{2A}R$ and $A_{2B}R$ (Meno and others 2001; Ngai and others 2001), while a tolerance in A_1R to caffeine might contribute to the absence of changes in SWA. Notably, chronic caffeine treatment has been found to strengthen $A_{2A}R$ agonism, which can downregulate the affinity of A_1R to caffeine through A_{2A}-A_1R heteromers (Ciruela and others 2006; Ferre and others 2008), therefore, potentially resulting in divergent responses modulated by each type of receptors.

To investigate the effects of daily caffeine intake on human GM, we used MRI and the classical structural MRI analyses. Within these analyses, differences in the outcome, the so-called GM volume, could also be associated with changes non-neuronal cells and/or in cerebral vasculature (Zatorre and others 2012). Such contributions seem not unlikely, as caffeine has been applied to induce apoptosis of glial cells in oncological studies (Li and others 2017). Furthermore, the effects of adenosine and its A_{2A} receptors on the release of growth factors have been strongly suggested (Cunha 2016), which in turn modulate the proliferation of astrocytes and can influence angiogenesis (Fredholm 2007). A direct evidence on the effect of daily caffeine intake, however, remains to be clarified.

An MRI-derived observation on GM changes can also be simply confounded by differences in CBF (Field and others 2003; Ge and others 2017; Laurienti and others 2003). Accordingly, in the present study, CBF accounted for *total* GM changes induced by caffeine intake. These apparent *total* GM changes emphasize the importance of controlling for caffeine consumption in repeated MRI measures, especially in the primarily impacted regions – cuneus, precuneus, cerebellum, and subcortices. In addition to the typical caffeine-induced reductions in CBF (Field and others 2003; Merola and others 2017; Vidyasagar and others 2013), our study adds that both differences in CBF and their impact on the apparent change of morphometry still remains after 5.5 hours after the caffeine intake.

Interestingly, while we observed slower reaction time and compromised accuracy in a visual memory task during daily intake, people who had higher caffeine and paraxanthine levels (as indicated by AUC-CAPX) within the caffeine condition showed faster reaction time than those with lower levels. This phenomenon might implicate two possibilities: a) since the between-subject variance of AUC-CAPX may also reflect traits in metabolism, it could also be that different metabolic patterns moderate the magnitude of caffeine's negative impact on working memory performance; or b) an acute higher concentration of caffeine and paraxanthine could still temporarily mitigate the worsened reaction from the long-term caffeine intake to a limited degree.

Notably, we did not find a significant association between *mTL* volume and the performance in visual working memory. N-back performance, as the only available measure in memory functions in the current project, might however not be a suitable task to detect neurocognitive changes related to *mTL* GM (Jeneson and Squire 2011). In fact, performing N-back task involves majorly frontal and parietal regions (Wang and others 2019). Moreover, a change in brain structure does not necessarily come with a difference in behavior, as differential brain activity patterns can compensate or adapt to structural changes such that behavioral performance remains unchanged (Barulli and Stern 2013; Rudrauf 2014). Therefore, we strongly encourage a further investigation in functional activities during working memory as well as other cognitive domains under daily caffeine consumption.

Increasing evidence suggests that moderate caffeine intake is beneficial and reduces the risk to develop several chronic diseases(van Dam and others 2020). Thus, it is of importance to note that our findings on cerebral structures and visual working memory were derived from young, healthy participants in a "neutral circumstance" (i.e. no manipulation of cognitive or physical states). This may set a different basis when comparing to the existing evidence. Evidence from a number of randomized controlled-trials (RCTs) consistently supported *acute* enhancement in vigilance, especially during compromised cognitive or physiological states. In respective of habitual intake, a few longitudinal cohorts revealed, in habitual caffeine consumers older than 65 years old that the years of prior caffeine intake was associated with less age-related cognitive decline (Arab and others 2011; Ritchie and others 2007; van Gelder and others 2007; Vercambre and others 2013) or a reduced risk for dementia and Alzheimer's disease (Eskelinen and others 2009; Lindsay and others 2002; Tomata and others 2016). However, other longitudinal cohort studies did not confirm these findings (Feng and others 2018; Feng and others 2012; Gelber and others 2011; van Boxtel and others 2003). Moreover, a recent meta-analysis (Zhou and others 2018) on 415,530 participants collected from international databases reported no associations between cognitive functions (memory and overall capacity) and self-reported lifetime caffeine intake or genetic variants linked with caffeine intake. Finally, RCTs investigating repeated intake in healthy population under neutral circumstance did not observe benefits in various cognitive tasks (Galduróz and Carlini 1996; Judelson and others 2005; Weibel and others 2019; Wing 1990). Therefore, the positive association between habitual caffeine intake and cognitive outcomes found in some of the observational studies might not necessarily refer to a causality. Nevertheless, neuroprotective effects of caffeine have been cumulatively reported in animals, especially in ameliorating symptoms of Parkinson's disease and preventing or normalizing cognitive decline in Alzheimer's models (Arendash and others 2009; Arendash and others 2006; Chen 2014; Cunha 2016). Therefore, we strongly suggest more longitudinal RCTs to investigate the neurocognitive outcomes of habitual caffeine intake in stratified population (e.g. by genetic trait, age, sex, in healthy participants or in patients) to characterize the adequate applications that can bring most benefits and least harm.

Our study also bears some limitations that require a careful interpretation. First, we had a relatively small sample size. However, we minimized the individual variances by the within-subject design, and strictly controlled for the influence of timing and the environment. We also implemented an ambulatory phase in each condition to ensure the participant entering the laboratory phase with a standardized condition. We also coped with this potential limitation in the analyses, by employing non-parametric permutation tests in all the voxel-based analyses. Secondly, one might argue that the absence of a significant difference in SWA might be due to a genetically predisposed insensitivity to caffeine of the selected population (Retey and others 2007). However, as the withdrawal from daily intake of 450 mg of caffeine has been shown to induce clear-cut responses in the sleep-homeostatic regulation of these participants (Weibel and others 2019), it seems unlikely that the selected population was insensitive to the stimulant. Notably, the habitual amount was calculated from all types of caffeinated diets, not only coffee intake but also tea, chocolate drinks, energy drinks, soda, and so on.

Overall, our findings derived from this the double-blind randomized cross-over laboratory study yield an insight on the *mTL* GM plasticity induced by the repeated intake of caffeine in a long-term course. As caffeine effects might differ with respect to the pattern of intake (sporadically vs. daily) and the brain's state (healthy vs diseased), we call for more well-controlled clinical trials to investigate the potential of caffeine to affect human brains and cognition. This might clarify the conditions for both detrimental and beneficial impacts of caffeine, a freely available psychostimulant all over the world.

Acknowledgement

We sincerely appreciate the contribution of our M.Sc. student Rowena Waldis, interns Andrea Schumacher, Laura Tincknell, M.Sc. student Sven Leach, and all the study helpers assisting in the experiment. We also thank Dr. med. Corrado Garbazza and Dr. med. Helen Slawik for the health check during screening process. We are grateful to the Core Facility of Mass Spectrometry at the Faculty of Chemistry, and the Joint Metabolome Facility, members of the Vienna Life-Science Instruments (VLSI). We especially appreciate all our participants for their volunteering and cooperation. This work is supported by the Swiss National Science Foundation (grant number: 320030-163058).

Author Contributions

Y.-S. L.: acquisition and analysis of data; drafting the manuscript and figures

J. W.: acquisition and preprocessing of data

H.-P. L.: conception of the study

F. S.: design of the study

M. M.: acquisition of the data

J. B.: acquisition of data

S. M.-M.: acquisition of data

C. G.: acquisition of data

S. B.: design of the study and data analysis

C. C.: conception and design of the study

C. R.: conception and design of the study; drafting the manuscript

Conflicts of Interest

No conflicts of interest to declare.

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