

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

A multimodal double-blind randomized-controlled trial

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1 **Introduction**

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

14 Disturbed sleep homeostasis can cause not only cerebral micromorphometric alterations in the mitochondria
15 and chromatin that leads to cell death (Abushov 2010; Zhao and others 2016; Zhao and others 2017), but
16 also macrostructural changes. Lower grey matter (GM) volumes were observed during abnormally high
17 sleep pressure, such as during sleep deprivation or sleep fragmentation. Liu and others (2014) reported
18 reduced thalamic GM volume along with impaired cognitive performance in healthy adults after 72-hour
19 prolonged waking compared to baseline. Dai and others (2018) demonstrated a GM dynamic through the
20 36-hour course of sleep deprivation, where a decrease in right thalamus, right insula, right inferior parietal
21 lobe, and bilateral somatosensory association cortex was observed by 32 hours of sleep deprivation. In
22 clinical studies, GM volume and cortical thickness were reduced in patients with various sleep disorders
23 (e.g. chronic insomnia (Altena and others 2010; Joo and others 2013), sleep apnea (Baril and others 2017),
24 and narcolepsy (Joo and others 2011)) compared to healthy controls. Together, the micro- and macro-
25 morphometric changes in GM in response to sleep deprivation might reflect a disrupted adenosine-

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

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

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

1 **Methods**

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

5 Recruitment and Participants

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

- 8 • self-reported caffeine intake <300 or >600 mg/day (calculations were based on the (Bühler and
9 others 2013), adapted according to a classification of (Snel and Lorist 2011) to ensure the safety
10 of caffeine intake and to exclude extreme response
- 11 • bad sleep quality, i.e. PSQI > 5 in the last four weeks assessed by the Pittsburg Sleep Quality
12 Index (PSQI) to control for sleep disturbances
- 13 • extreme chronotype, as defined by Horne-Ostberg's Morningness-Eveningness Score (HOMES)
14 ≤ 30 or ≥ 70 to prevent pronounced variance in circadian phase.
- 15 • self-reported regular substance use (including medication, nicotine, and drugs) and other major
16 medical conditions.

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

22 Participants, Study Protocol and Environmental Control

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

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

3 Each protocol consisted of 9 days of ambulatory phase, followed by the strictly controlled laboratory stay
4 (**Fig 1**). In order to examine the effects from daily caffeine intake and to avoid withdrawal effects during
5 the laboratory phase at abstinence (Juliano and Griffiths 2004), the treatment, caffeine (3 x 150 mg/day) or
6 placebo (mannitol) capsules, had been administered for 9 days prior to the laboratory phase. Timing of
7 intake was set to 45 minutes, 4 hours, and 8 hours daily after waking up to imitate the pattern of caffeine
8 intake in reality (Martyn and others 2018). During the 9 days of ambulatory phase, participants complied
9 to a fixed sleep-wake cycle (8 hours \pm 30 minutes in bed, no naps allowed) to control for high sleep debt.
10 Individual bedtimes were chosen according to usual bedtimes of each participant (Tinguely and others
11 2014). The compliance to the sleep schedule was monitored by actimetry and sleep diaries. Furthermore,
12 participants were asked to abstain from caffeine-containing diets including coffee, tea, energy drink, soda,
13 and chocolate, etc. To check for compliance to the treatments, participants were instructed to collect
14 fingertip sweat samples during the ambulatory phase with standard cleaning procedure every evening 2
15 hours before bedtime. Sweat from the fingertips is an emerging tool for metabolomic biomonitoring in
16 humans (Brunmair and others 2020).

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

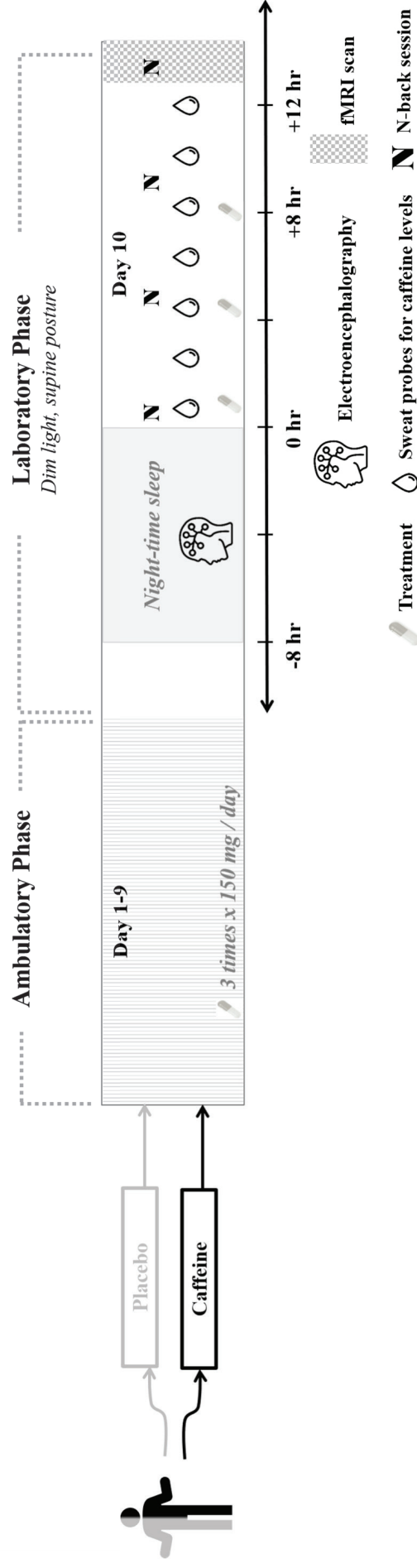
22 On the 10th day after waking up, the treatment (caffeine vs placebo) continued at identical times as during
23 the ambulatory phase (see **Fig 1**). The time of the protocol was adapted to individual's habitual bedtime. A
24 comparison of salivary dim-light melatonin onset (DLMO) did not indicate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

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

5

Uncorrected Accepted Version

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.



1 Data acquisition

2 1. MRI

3 T1-weighted structural data were obtained with a three-dimension (3D) magnetization-prepared rapid
4 gradient-echo (MP-RAGE) sequence (1x1x1mm³, TR=2000ms, TI= 1000ms, TE=3.37ms, FA=8°) on
5 a 3T Siemens scanner (Prisma; Siemens AG, Erlangen, Germany). Cerebral blood flow (CBF) was
6 measured by 2D-echo-planar imaging pulsed arterial spin labeling (ASL) sequence (4x4x4mm³,
7 TR=3000ms, TE=12ms, FA=90°, FoV=100) at the same MR scanner.

8 2. Sleep EEG

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

16 3. Caffeine and metabolites from fingertip sweat

17 Considering the inter-individual variance in caffeine metabolism (Nehlig 2018), we measured the
18 individual levels of caffeine and caffeine metabolites in the sweat from the fingertips during the
19 ambulatory and laboratory phases. Samples were collected once daily during the ambulant phase, while
20 the laboratory phase included 7 times repeated sampling in 2-hour intervals from the awakening until
21 the MRI scan. Sweat was collected using 1 cm² sampling unit. Samples were then processed and
22 analyzed according to Brunmair and others (2020). In short, the metabolites were extracted from the
23 sampling unit using an acidified aqueous solution and subjected to analysis by liquid chromatography-
24 mass spectrometry (LC-MS) using a Q Exactive HF orbitrap hyphenated with a Vanquish UHPLC

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

3 4 4. N-back task

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

15 16 Data Preprocessing and Analyses

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

22 1. MRI data for GM and CBF

23 For the preprocessing of T1-weighted images, we used CAT12, an extension toolbox of SPM12
24 (University College London, London, UK). We adopted the built-in pipeline for repeated measures,
25 where volumetric measures were co-registered to the mean of the two volumes from each participant.

26 Total intracranial volume (TIV) and the volumes of each cerebral compartment (grey matter, white

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

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

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

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

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

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

6 2. EEG slow wave activity

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

20 3. Caffeine and paraxanthine levels

21 We kept all values original including the ones below limit of detection. To estimate the individual
22 amount of caffeine exposure, we combined the area under the curves of caffeine (CA) and paraxanthine
23 (PX), abbreviated CAPX, as paraxanthine also antagonizes adenosine at A₁, A_{2A}, and A_{2B} receptors
24 (Cornelis and others 2016), while it follows a similar but slower metabolic pattern as compared to
25 caffeine. To investigate the association of acute caffeine and paraxanthine exposure with other variables

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

4 4. N-Back

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

12

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15 **Results**16 *Caffeine induces a decrease in grey matter (GM) volume*

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

21 *Total* GM volume was lower in the caffeine condition compared to placebo ($t = -3.59$; $p < .001$; 95% CI of
22 coefficients = [-0.009, -0.003]; $R^2_m = 0.75$; $R^2_c = 0.97$, **Fig 2**). A voxel-based analysis (VBA) indicated
23 that the reduction of GM volume was most prominent in the right medial temporal lobe (*mTL*, including
24 hippocampus; VBA threshold: cluster level $p_{FWE} < .05$, **Fig 3**), along with the following regions at trend (i.e.
25 $0.05 \leq p_{FWE} < .063$) of a reduction: left frontal pole, right postcentral gyrus, right insula, and the cerebellum.
26 Both *total* GM and *mTL* GM (**Fig 4**) were negatively associated with AUC-CAPX, i.e. the larger the
27 reduction was in GM the higher was the individual AUC-CAPX (statistics see **Table 1**). No significant
28 increases of GM in the caffeine compared to the placebo condition were observed.

29

30

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 $\text{response}_{\text{caff or plac}} - (\text{response}_{\text{caff}} + \text{response}_{\text{plac}})/2$. 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.

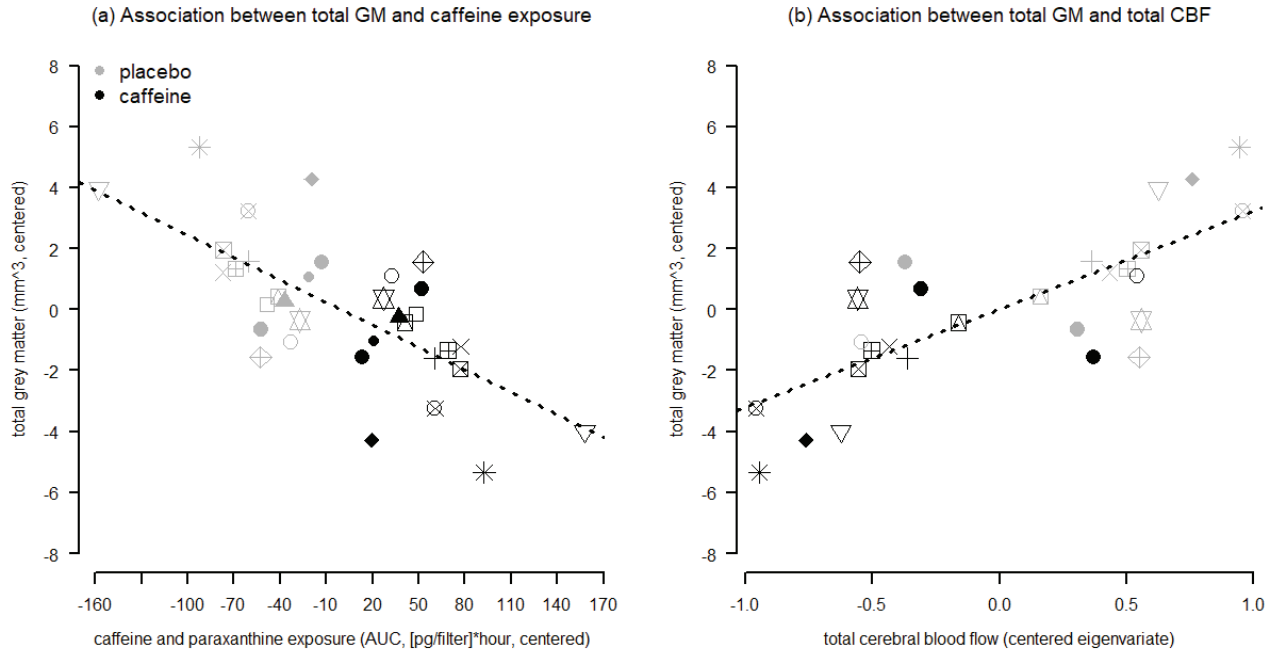


Figure 3. Reduction in medial temporal grey matter. The blue area indicated the clusters that showed a significant GM reduction in medial temporal lobe (at combined voxel-cluster-level $p_{\text{uncorrected}} < 0.001$, $p_{\text{FWE}} = .032$) in the caffeine condition compared to placebo, based on a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

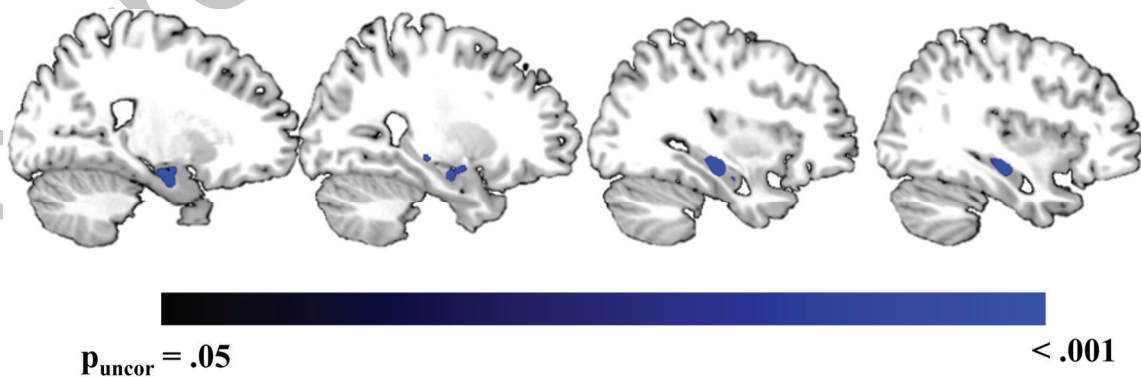
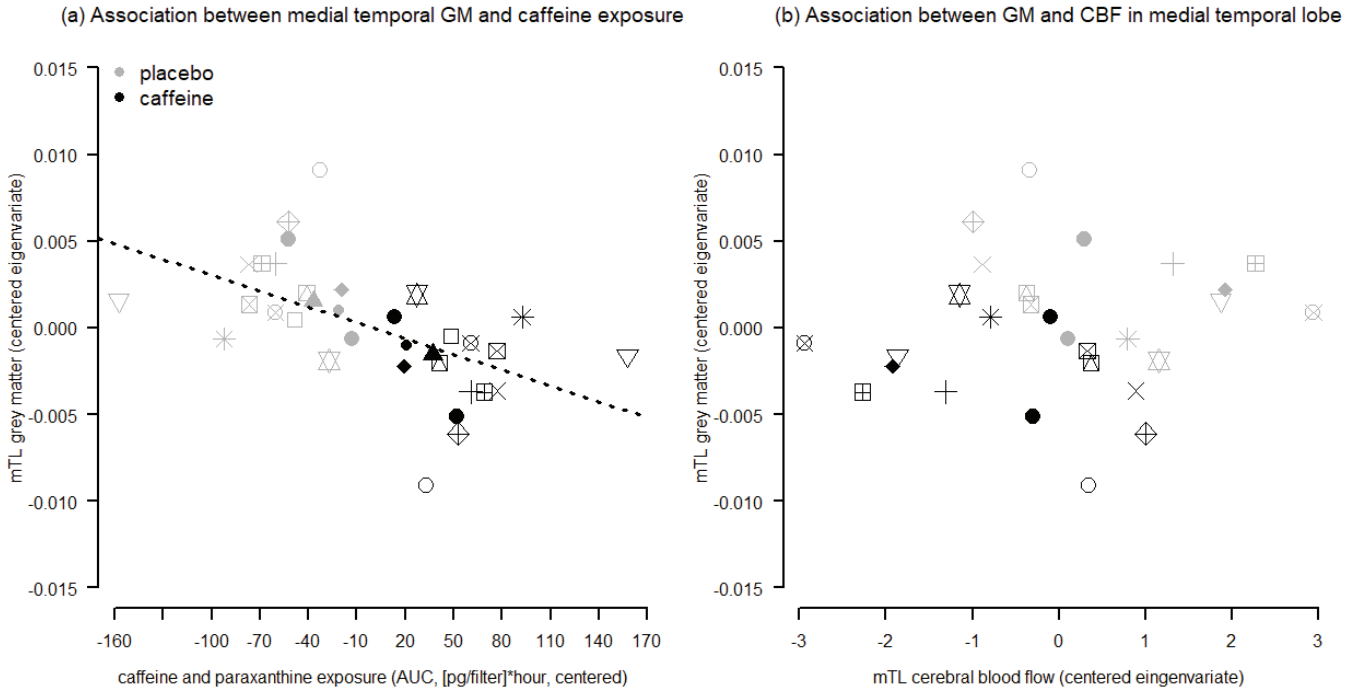


Figure 4. The associations of medial temporal grey matter volumes with medial temporal (*mTL*) cerebral blood flow and caffeine metabolites.

(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.



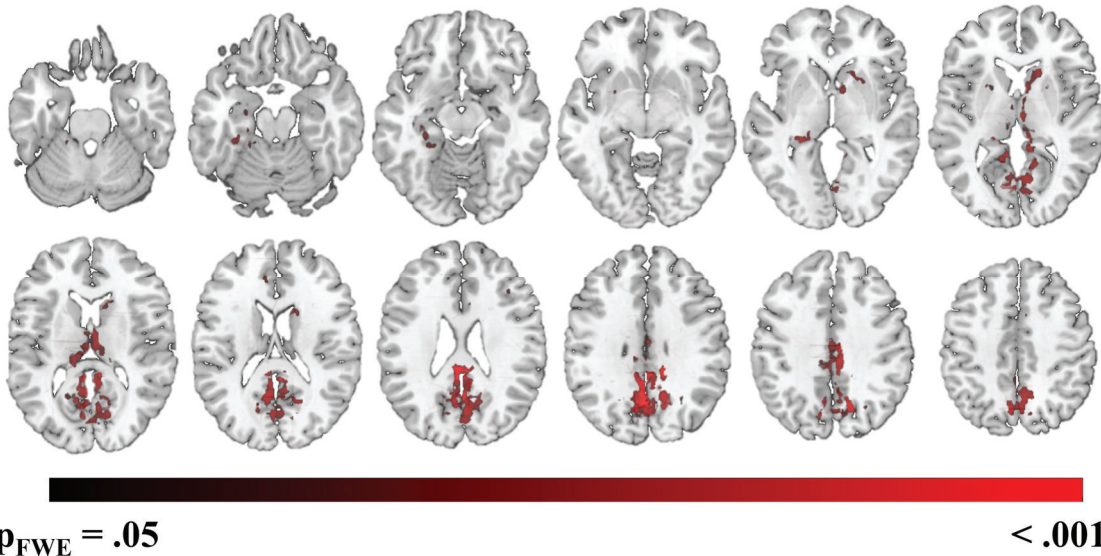
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1 ***Cerebral blood flow (CBF) remains reduced at 5.5 hours after the last caffeine intake***

2 Compared to the placebo condition, *Total CBF* was significantly reduced in the caffeine condition ($t = -5.2$;
 3 $p < .001$; 95% CI of coefficients = $[-0.119, -0.054]$; $R^2_m = 0.12$; $R^2_c = 0.71$). Voxel-wise analysis suggested
 4 that the reductions occurred prominently in the midline, including cuneus, precuneus, and subcortical
 5 regions (cluster level $p_{FWE} < .05$) (**Fig 5**). AUC-CAPX was negatively associated with both *total CBF* and
 6 *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.



7

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

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

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

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

5 In NREM SWA we did not observe significant differences between caffeine and placebo conditions ($t = -$
 6 8.87 , $p = .0386$). However, NREM SWA was positively associated with *total* GM, and a significant
 7 interaction between condition and SWA indicated a stronger association in the placebo condition. No
 8 significant associations between SWA and *mTL* GM volumetric reductions were seen (**Table 1**).

9 ***Exploratory analyses indicate poorer working memory performance during daily caffeine intake***

10 As an exploratory step to observe whether a change in the memory domain might be present concomitantly
 11 with the *mTL* GM changes, we tentatively inspected into the only memory-related assessment (N-back)
 12 conducted in the study. The inter-individual variance of the reductions in response accuracy was not
 13 associated with the magnitude of *total* nor *mTL* GM reduction, however, a relatively poorer working
 14 memory performance was found in caffeine condition: Compared to placebo, lower accuracy was found in
 15 both 0-back ($t = -2.33$, $p = .020$) and 3-back ($t = -2.02$, $p = .044$) performance in the caffeine condition, as
 16 well as a lower *net* accuracy, i.e. response accuracy in 3-back corrected for the baseline response in 0-back
 17 performance ($t_{\text{con}} = -1.97$, $p_{\text{con}} = .049$; $R^2_{\text{m}} = .41$, $R^2_{\text{c}} = .85$). Furthermore, the RT of missed ($t_{\text{con}} = 2.75$,
 18 $p_{\text{con}} = .006$; $R^2_{\text{m}} = 0.87$; $R^2_{\text{c}} = 0.99$) and correction rejections ($t_{\text{con}} = 1.95$, $p_{\text{con}} = .051$; $R^2_{\text{m}} = 0.90$; $R^2_{\text{c}} =$
 19 0.99) were longer in caffeine condition compared to placebo, albeit no difference in the overall and in hits
 20 and false alarm responses ($t_{\text{all}} < 1.88$, $p_{\text{all}} > .60$). Within the caffeine condition, a higher AUC-CAPX level
 21 was associated with a better *net* accuracy ($t_{\text{AUC}} = 3.58$, $p_{\text{AUC}} < .001$; $R^2_{\text{m}} = 0.87$; $R^2_{\text{c}} = 0.99$) and a shorter *net*
 22 RT in all types of responses ($t_{\text{all}} < -2.44$, $p_{\text{all}} < .015$; $R^2_{\text{m}} = 0.87$; $R^2_{\text{c}} = 0.99$).

23

24

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^2 margian], R^2 conditional]). The R^2 marginal includes only the variance of the fixed factors, while R^2 condition includes the variance of all (fixed + random factors).

		<i>Dependent variables</i>				
		<i>Total GM</i>	<i>mTL GM</i>	<i>Total CBF</i>	<i>mTL CBF</i>	<i>SWA</i>
<i>Independent variables</i>	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 $\bar{\tau}$ 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]	--

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

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

9 $\bar{\tau}$ Including total CBF as a covariate fully accounted for the main effect of condition on total GM estimation ($t_{CBF} = 2.82$, $p_{CBF} = .005$; $t_{con} = -0.77$, $p_{con} = .441$).

1 Discussion

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

15 Based on the evidence of sleep-homeostatic effects on brain morphology (Dai and others 2018; Liu and
16 others 2014), we assumed that daily caffeine intake may consequently affect GM volumes. While the data
17 indeed support the latter, this observed alteration did not result from a proportionate disturbance in
18 homeostatic sleep pressure during nighttime and thus may suggest another mechanism. While caffeine
19 reinstates the adenosine-inhibited synaptic excitatory signaling in human neurons (Kerkhofs and others
20 2017) and attenuates synaptic long-term potentials (Costenla and others 2010; Lopes and others 2019),
21 SWA during sleep mirrors the renormalization of synaptic capacity and recovery of brain neurons from the
22 energy consumption during prior wakefulness (Tononi and Cirelli 2003; 2014). We speculate that, between
23 the energy usage during wakefulness and the recovery of neurons at sleep, daily caffeine intake perhaps did
24 not slant the balance by compromising the side of recovery process but raising the side of energy usage. In
25 other words, an enhanced excitation in neurons induced by caffeine might increase the *need* of synaptic

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

4 A few reasons might account for the co-occurrence of a caffeine-induced response in CBF and the absence
5 of any clear-cut changes in SWA. First of all, there was a difference in timing of measurements. CBF was
6 measured at 5.5 hours after the last intake, while the start of nighttime sleep was set to 8 hours after the last
7 intake. The previous evidence with different durations from the last intake suggests that a longer duration
8 may attenuate the effect of caffeine on sleep (Drapeau and others 2006; Landolt and others 1995a; Landolt
9 and others 1995b). Moreover, different subtypes of adenosine receptors (A_1R and $A_{2A}R$) may mediate
10 caffeine-induced changes in CBF and SWA. A_1R has much stronger propensity to develop a tolerance to
11 daily caffeine treatment compared to $A_{2A}R$ (Halldner and others 2000; Johansson and others 1993;
12 Johansson and others 1997; Karcz-Kubicha and others 2003; Popoli and others 2000). Thus, daily caffeine
13 intake remains its effect in CBF through the blockade of $A_{2A}R$ and $A_{2B}R$ (Meno and others 2001; Ngai and
14 others 2001), while a tolerance in A_1R to caffeine might contribute to the absence of changes in SWA.
15 Notably, chronic caffeine treatment has been found to strengthen $A_{2A}R$ agonism, which can downregulate
16 the affinity of A_1R to caffeine through A_{2A} - A_1R heteromers (Ciruela and others 2006; Ferre and others
17 2008), therefore, potentially resulting in divergent responses modulated by each type of receptors.

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

1 An MRI-derived observation on GM changes can also be simply confounded by differences in CBF (Field
2 and others 2003; Ge and others 2017; Laurienti and others 2003). Accordingly, in the present study, CBF
3 accounted for *total* GM changes induced by caffeine intake. These apparent *total* GM changes emphasize
4 the importance of controlling for caffeine consumption in repeated MRI measures, especially in the
5 primarily impacted regions – cuneus, precuneus, cerebellum, and subcortices. In addition to the typical
6 caffeine-induced reductions in CBF (Field and others 2003; Merola and others 2017; Vidyasagar and others
7 2013), our study adds that both differences in CBF and their impact on the apparent change of morphometry
8 still remains after 5.5 hours after the caffeine intake.

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

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

1 Increasing evidence suggests that moderate caffeine intake is beneficial and reduces the risk to develop
2 several chronic diseases(van Dam and others 2020). Thus, it is of importance to note that our findings on
3 cerebral structures and visual working memory were derived from young, healthy participants in a “neutral
4 circumstance” (i.e. no manipulation of cognitive or physical states). This may set a different basis when
5 comparing to the existing evidence. Evidence from a number of randomized controlled-trials (RCTs)
6 consistently supported *acute* enhancement in vigilance, especially during compromised cognitive or
7 physiological states. In respective of habitual intake, a few longitudinal cohorts revealed, in habitual
8 caffeine consumers older than 65 years old that the years of prior caffeine intake was associated with less
9 age-related cognitive decline (Arab and others 2011; Ritchie and others 2007; van Gelder and others 2007;
10 Vercambre and others 2013) or a reduced risk for dementia and Alzheimer’s disease (Eskelinen and others
11 2009; Lindsay and others 2002; Tomata and others 2016). However, other longitudinal cohort studies did
12 not confirm these findings (Feng and others 2018; Feng and others 2012; Gelber and others 2011; van
13 Boxtel and others 2003). Moreover, a recent meta-analysis (Zhou and others 2018) on 415,530 participants
14 collected from international databases reported no associations between cognitive functions (memory and
15 overall capacity) and self-reported lifetime caffeine intake or genetic variants linked with caffeine intake.
16 Finally, RCTs investigating repeated intake in healthy population under neutral circumstance did not
17 observe benefits in various cognitive tasks (Galduróz and Carlini 1996; Judelson and others 2005; Weibel
18 and others 2019; Wing 1990). Therefore, the positive association between habitual caffeine intake and
19 cognitive outcomes found in some of the observational studies might not necessarily refer to a causality.
20 Nevertheless, neuroprotective effects of caffeine have been cumulatively reported in animals, especially in
21 ameliorating symptoms of Parkinson’s disease and preventing or normalizing cognitive decline in
22 Alzheimer’s models (Arendash and others 2009; Arendash and others 2006; Chen 2014; Cunha 2016).
23 Therefore, we strongly suggest more longitudinal RCTs to investigate the neurocognitive outcomes of
24 habitual caffeine intake in stratified population (e.g. by genetic trait, age, sex, in healthy participants or in
25 patients) to characterize the adequate applications that can bring most benefits and least harm.

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

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

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27

28 **Author Contributions**

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

30 J. W.: acquisition and preprocessing of data

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

32 F. S.: design of the study

33 M. M.: acquisition of the data

34 J. B.: acquisition of data

35 S. M.-M.: acquisition of data

36 C. G.: acquisition of data

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

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40

41 **Conflicts of Interest**

42 No conflicts of interest to declare.

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