

# Sleep restriction alters plasma endocannabinoids concentrations before but not after exercise in humans



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## ABSTRACT

Following binding to cannabinoid receptors, endocannabinoids regulate a variety of central nervous system processes including appetite and mood. Recent evidence suggests that the systemic release of these lipid metabolites can be altered by acute exercise and that their levels also vary across the 24-h sleep-wake cycle. The present study utilized a within-subject design (involving 16 normal-weight men) to determine whether daytime circulating endocannabinoid concentrations differ following three nights of partial sleep deprivation (4.25-h sleep opportunity, 2:45–7 a.m. each night) vs. normal sleep (8.5-h sleep opportunity, 10:30 p.m.–7 a.m. each night), before and after an acute bout of ergometer cycling in the morning. In addition, subjective hunger and stress were measured. Pre-exercise plasma concentrations of 2-arachidonoylglycerol (2AG) were 80% higher 1.5 h after awakening (vs. normal sleep,  $p < 0.05$ ) when participants were sleep-deprived. This coincided with increased hunger ratings (+25% vs. normal sleep,  $p < 0.05$ ). Moreover, plasma 2AG was elevated 15 min post-exercise (+44%,  $p < 0.05$ ). Sleep duration did not however modulate this exercise-induced rise. Finally, subjective stress was generally lower on the day after three nights of short sleep vs. normal sleep, especially after exercise ( $p < 0.05$ ). Given that activation of the endocannabinoid system has been previously shown to acutely increase appetite and mood, our results could suggest that behavioral effects of acute sleep loss, such as increased hunger and transiently improved psychological state, may partially result from activation of this signaling pathway. In contrast, more pronounced exercise-induced elevations of endocannabinoids appear to be less affected by short sleep duration.

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## 1. Introduction

The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2AG) are endogenously produced lipids that have been found, for instance, to be elevated in blood in response to psychological stress (Hill et al., 2009; Dlugos et al., 2012). Due to their highly lipophilic properties, endocannabinoids can readily cross the blood–brain barrier. Following their binding

to central nervous system receptors (e.g. CB1 receptors), endocannabinoids have been shown, e.g., to reduce anxiety (Marsicano et al., 2002; Lutz et al., 2015) and increase food intake (Di Marzo et al., 2001). In relation to the latter, recent findings suggest 2AG to be more linked to hedonic motivation for food whereas AEA seems to be more involved in driving homeostatic eating (Monteleone et al., 2016). Remarkably, the aforementioned effects of endocannabinoids resonate with those of sleep restriction, an increasingly prevalent stressor in modern society (Ford et al., 2015). Acute sleep loss increases food intake and ideal portion sizes (Hogenkamp et al., 2013; St-Onge et al., 2011), accentuates the brain's hedonic response to food (Benedict et al., 2012), and relatedly, impairs the ability to cognitively inhibit pre-potent

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responses to rewarding food stimuli (Cedernaes et al., 2014; topics reviewed in Cedernaes et al., 2015a). Moreover, similar to the anxiolytic effects of endocannabinoids, acute sleep deprivation has repeatedly been shown to acutely alleviate symptoms of depression (Wu et al., 1999). However, to date, only two studies have been able to link systemic release of plasma concentrations of endocannabinoids to acute sleep loss in humans (Hanlon et al., 2016; Vaughn et al., 2010), and have not tested how further physiological stress impacts this signaling system.

Another stressor that has been shown to activate the endocannabinoid system is physical exercise (Dietrich and McDaniel, 2004). For instance, a recent study involving 11 healthy trained male cyclists found plasma AEA concentrations to be increased during and shortly after intense exercise (i.e., 60 min at 55% followed by 30 min at 75%  $W_{max}$ ; Heyman et al., 2012). In another study, treadmill running resulted in increased blood levels of AEA; however, only at moderate – versus low or high – exercise intensity (i.e., at 70–85% of age-adjusted maximum heart rate) (Raichlen et al., 2013). Finally, a study involving 24 young males demonstrated that 1 h of moderate intensity exercise increased plasma AEA concentrations. In the same study, plasma 2AG concentrations showed a similar trend to increase following exercise, but failed to reach statistical significance (Sparling et al., 2003). To our best knowledge, no study has yet investigated whether exercise-induced elevations of endocannabinoids are affected by short sleep duration. Exercise-induced elevations of endocannabinoid levels have been proposed to account partially for anxiolytic (Tantimonaco et al., 2014; Fuss et al., 2015), anti-nociceptive (Galdino et al., 2014) and neuro-protective (Hill et al., 2010a) properties of exercise, which are all responses that instead are affected in a negative direction by sleep loss (e.g. Roy-Byrne et al., 1986; Schuh-Hofer et al., 2013; Cedernaes et al., 2016).

Against this background, the present study, involving 16 healthy normal-weight men, sought to determine whether short sleep vs. normal sleep duration would alter circulating levels of different endocannabinoid species, before and after an acute bout of ergometer cycling. In addition, subjective stress and hunger ratings were measured. Given that increased systemic release of plasma concentrations of endocannabinoids has recently been linked to acute sleep loss, we expected recurrent sleep loss to alter plasma endocannabinoid concentrations. Moreover, we hypothesized that exercise would transiently increase plasma endocannabinoid concentrations.

## 2. Material and methods

### 2.1. Participants

Sixteen male non-smokers were included in the present study [age:  $22.9 \pm 0.66$  years; BMI:  $22.9 \pm 0.46$  kg/m<sup>2</sup>]. An anamnestic interview by a medical doctor (J.C.) before study inclusion ensured that participants were in general good health (also verified by clinical blood parameters, e.g. white blood cell count), not on medication, and that none of them reported to have been ever been diagnosed with or treated for a psychiatric condition. This evaluation included general questions regarding current and prior depressive, anxiety or other psychiatric symptoms, heredity, as well as possible current or prior use of medications or dietary/pharmaceutical supplements, including specific questions about psychiatric medications. Answers were also confirmed and further elaborated during an anamnestic interview prior to study inclusion (conducted by J.C.). Only subjects who stated during screening that they habitually go to bed between 10 p.m. and 12 p.m., and who slept 7–9 sleep hours per night, were included in the present study.

All participants provided written informed consent. The study was conducted in accordance with the Helsinki Declaration and was approved by the Regional Ethical Review Board in Uppsala (EPN 2014/242/1).

### 2.2. Experimental procedure

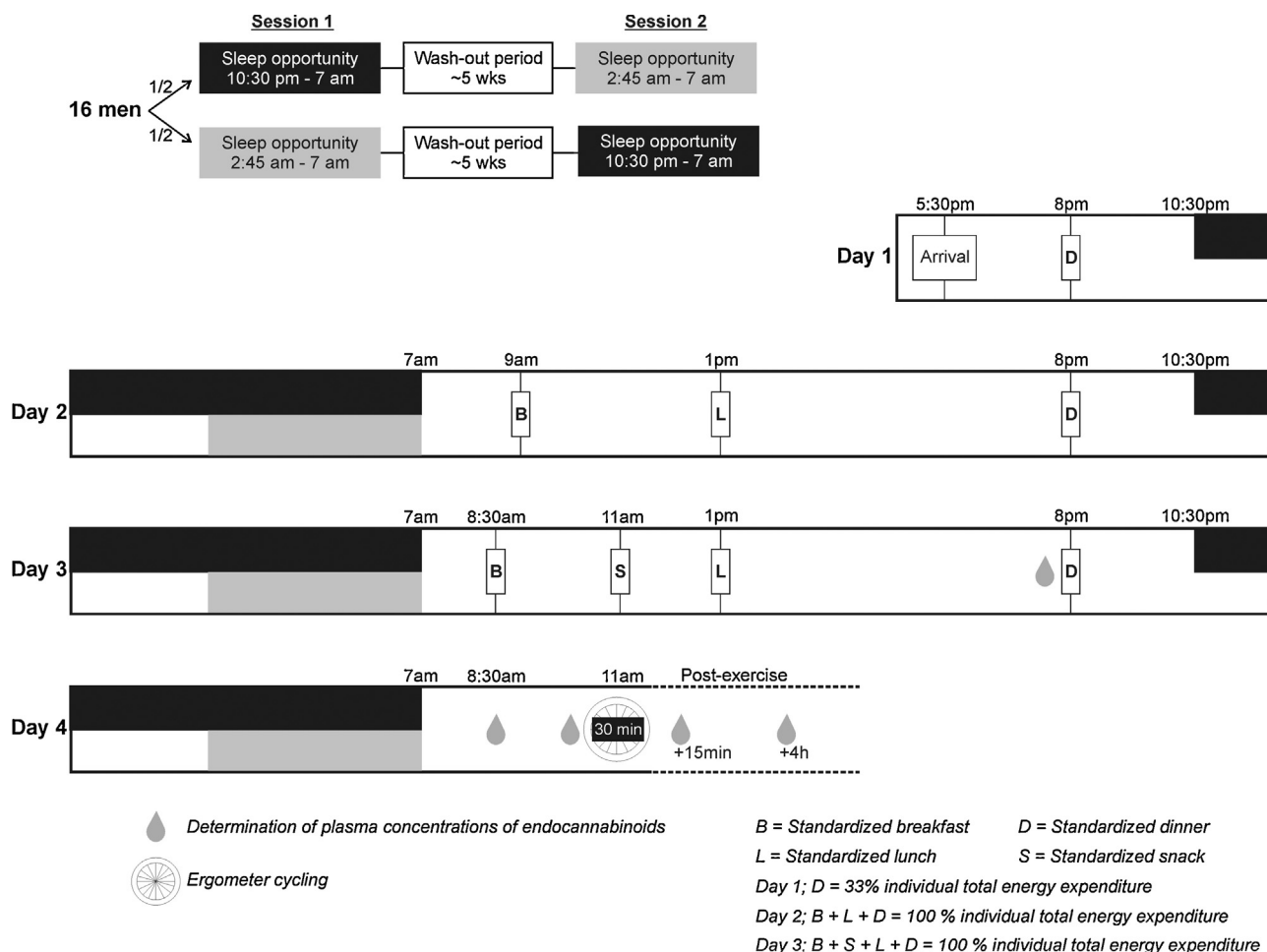
The study was based on a within-subject design, meaning that all participants took part in two separate experimental conditions (normal sleep vs. short sleep duration), each of which lasted four consecutive days and three nights (day and night 1; day and night 2; day and night 3; day 4) in our sleep laboratories at Uppsala University. The order of experimental conditions was randomized and counterbalanced across subjects (Fig. 1).

In each experimental condition, upon arrival at 5:30 p.m. on the baseline day (day 1), participants were provided with a standardized dinner (8 p.m.), and were then prepared for sleep polysomnography. In the normal sleep condition, participants were given an 8.5-h sleep opportunity (10:30 p.m.–7 a.m.) on each experimental day. In the short sleep condition, sleep was allowed for 4.25 h each night (2:45–7 a.m.) on experimental days, with lights kept below 6 lx at eye level between 10:30 p.m. and 2:45 a.m.. During all experimental wake periods, i.e., when sleep was not scheduled, participants were allowed to spend their time with a selection of movies, games, and books, and they were continuously monitored by experimenters. On each experimental day, participants were provided with standardized meals, but were only allowed to drink water (see Fig. 1). The sum of calories provided by all meals on each experimental day (except for the first day where participants arrived in the late afternoon) corresponded to individual energy requirements (based on the Harris-Benedict equation factored 1.2 for light physical activity; calories on the first day corresponded to 33% of this calculated requirement). Participants were also provided with three supervised scheduled walks on day 2 and day 3 (daily total duration 60 min); otherwise they were confined to their rooms and could engage in sedentary-level activities. On day 4 (i.e., after 3 nights of either short or normal sleep), participants were kept fasted, enabling the investigation of the influence of sleep duration on fasting circulating concentrations of endocannabinoids, both before and after acute exercise (see Section 2.3).

On the day of the exercise intervention (day 4), self-reported hunger and stress were measured by means of a 100-mm visual analogue scale (with 0 representing “Not hungry at all”/“Not stressed at all” and 100 mm representing “Very hungry”/“Very stressed”) at the following time points: 8:30 a.m., ~10 a.m., ~11 a.m., and ~3 p.m.

### 2.3. Exercise protocol

Exercise intensity was calculated prior to the first experimental session for each individual by using a step-wise increasing ergometer evaluation test (the Åstrands-Ryhming nomogram method, with age-adjusting correction factors; Cink and Thomas, 1981). Based on the relationship between workload (in Watts), and heart rate and oxygen consumption, obtained during this test, the  $VO_2$  reserve capacity was calculated (which, in contrast to  $VO_2$  max, to a greater extent takes into account an individual's resting oxygen consumption level, and also corresponds more closely to heart rate reserve, another measure of exercise intensity; Swain and Leutholtz, 1997). The value of 75% of the  $VO_2$  reserve capacity was then converted to a corresponding workload value (in Watts), which was used for subsequent exercise tests on the same ergometer. The ergometer evaluation test took place 1–2 weeks before the first session, and all ergometer tests were carried out on a standard stationary ergometer (Lode Corival, Intramedic).



**Fig. 1.** Experimental scheme. Abbreviations: NS, normal sleep condition; PSD, partial sleep deprivation condition; wks, weeks; n = number of subjects.

The exercise protocol during the short and normal sleep conditions was 35 min long, and consisted of a 5-min warm-up period at 25% of the subsequent workload. The higher workload corresponded to 75% of  $\text{VO}_2$  reserve (average  $177 \pm 10$  W) and was maintained for 30 min after the warm-up period. Preceding to as well as after this exercise protocol on day 4, participants were bed-restricted and could only engage in sedentary activities.

#### 2.4. Determination of plasma concentrations of endocannabinoids

Blood samples were taken from an intravenously placed catheter at five different time points: day 3 at 7:30 p.m.; day 4 at 8:30 a.m., ~10 a.m., (followed by the 35-min exercise protocol), ~15 min and ~4 h after the end of the exercise protocol. Immediately after sampling with EDTA collection tubes, blood was centrifuged ( $4^\circ\text{C}$ , 10 min, 1300g), plasma was removed and then frozen at  $-80^\circ\text{C}$ .

Plasma levels of 2AG and AEA, and of the two AEA congeners [*N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA)] were measured according to a previously described method (Fanelli et al., 2012) from 250- $\mu\text{l}$  plasma samples. Given that 2AG is chemically unstable and undergoes rapid isomerization to 1AG, we also measured concentrations of 1AG. The sum of 1AG and 2AG was therefore used as a measure of biological 2AG levels in the present study.

Samples were rapidly thawed and spiked with deuterated internal standards before being extracted with 2 ml of toluene (Carlo

Erba RS HPLC grade, Rodano, Italy). After a 10-min vortex and centrifugation ( $4^\circ\text{C}$ , 10 min, 2000g), samples were dipped for 5 min into methanol that had been bath cooled with dry ice. The upper layer was transferred to  $12 \times 75$  mm Pyrex tubes (Bibby Scientific, Riozzo di Cerro, Italy) and dried under a nitrogen stream. Samples were reconstituted with 100  $\mu\text{l}$  MeOH, and 70  $\mu\text{l}$  of the resulting solution was injected into the LC-MS/MS system, composed of an HPLC Series 200 (PerkinElmer, Waltham, USA) and of an API 4000 QTrap instrument (AB-Sciex, Toronto, Canada). Samples underwent on-line purification on a fusion column (POROS R1/20 2.1  $\times$  30 mm, Applied Biosystems, Foster City, CA), separation by an analytical Discovery HS C18 3  $\mu\text{m}$  7.5  $\times$  4.6 mm column (Supelco, Bellefonte, PA) and detection by atmospheric pressure chemical ionization and multiple reaction monitoring mode. Quantitative and qualitative transitions were recorded for each analyte in order to ensure specificity. The assay functional sensitivity in plasma matrix was 0.039 pmol/ml for AEA, 0.156 pmol/ml for 2AG and 1AG, and 0.390 pmol/ml for PEA and OEA.

As previously described (Fanelli et al., 2012), endocannabinoids levels in blood or plasma undergo a rapid increase in a time-dependent manner, possibly because they are released by blood cells (AEA, PEA and OEA) or derived by lipid metabolism (2AG and 1AG). In order to minimize the analytical variability caused by this phenomenon, samples from the same subject were processed in the same batch, and each batch included two replicates of low and high level quality controls (QCs). Low level QCs were obtained by pooling plasma samples derived by rapid blood processing and storage, so that low physiologic endocannabinoids concentra-

tions were preserved. Conversely, high level QCs were obtained by pooling discarded plasma samples kept at 4 °C for 48 h, thus containing increased endocannabinoids levels. Each QC was parted in multiple individual aliquots. In each analytical batch, QC aliquots were strictly defrosted and processed as unknown samples. Intra-batch, intra-assay and inter-assay imprecision was, respectively, 0.5–11.2%, 2.0–13.9% and 0.6–17.1% for all analytes at low and high level.

### 2.5. Sleep assessment

Prior to the first experimental session, participants had an overnight polysomnography in our sleep laboratory to reduce possible bias from the first-night-effect on sleep maintenance and quality in subsequent experimental nights (see also Tamaki et al., 2016). Participants were considered eligible for inclusion into the present study only if sleep parameters during their adaption night were within the standard range (e.g. sleep onset latency less than 30 min). The anamnestic interview prior to study inclusion further ensured that none of the participants reported regular snoring, nor did they report regular excessive daytime sleepiness, and finally, they had never been diagnosed with any sleep disorder or psychiatric disorder. However, sleep polysomnography during the adaption night was not performed with e.g. respiratory recordings. Thus, although unlikely, we cannot completely rule out the possibility that some of our participants may have suffered from e.g. sleep disorder breathing, although the likelihood thereof is further reduced by the fact that we only studied normal-weight, young and healthy participants.

During experiments, sleep was measured by Embla A10 recorders (Flaga hf, Reykjavik, Iceland). EEG signals were derived from C3, C4, Fp1, Fp2 and referenced to the contralateral mastoid. Sleep was subsequently scored by an experienced scorer (J.E.B.) according to standard criteria (Silber et al., 2007).

### 2.6. Statistical analysis

Repeated measures ANOVA was conducted to investigate our two main study aims: (a) the influence of recurrent short sleep duration on fasting circulating concentrations of endocannabinoids (within-subject factors: *Sleep condition* and *Pre/Post-sleep* → day 3: 7:30 p.m., day 4: 8:30 a.m.) and (b) whether recurrent short sleep duration would alter the effects of acute physical exercise on circulating concentrations of endocannabinoids (within-subject factors: *Sleep condition* and *Exercise time point* → day 4: ~10 a.m. (last pre-exercise time point), as well as 15 min and 4 h post-exercise). Moreover, a repeated measures ANOVA was utilized to investigate (c) the effects of recurrent partial sleep loss vs. normal sleep on subjective hunger and stress ratings on day 4 (within-subject factors: *Sleep condition* and *Time* on day 4: 8:30 a.m., ~10 a.m., ~11 a.m., and ~3 p.m.). The Greenhouse–Geisser method was used to correct for sphericity deviations. Pearson's correlation analysis was utilized to examine putative relationships between circulating concentrations of endocannabinoids and rating scores at timepoints with coinciding blood measurements and subjective ratings of hunger and stress.

Overall, two-tailed *p*-values below 0.05 were considered significant. Data are presented as means ± standard error of the mean (SEM), unless otherwise stated.

## 3. Results

### 3.1. Sleep

A sleep diary that was kept by the participants confirmed that average self-reported sleep duration in the week before the start of

each experimental session did not differ between the short and normal sleep conditions (range 7–9 h per night; *p* > 0.10, as determined by a paired *t*-test).

In the normal sleep condition (i.e., where subjects could sleep for 8.5 h each night), the polysomnographic analysis revealed that subjects slept in total 7.95 ± 0.13 h on the first night, 7.91 ± 0.10 h on the second night, and 8.02 ± 0.10 h on the third night. In the short sleep condition (i.e., subjects had an sleep opportunity of 4.25 h each night), they slept in total 3.97 ± 0.05 h on the first night, 4.07 ± 0.03 h on the second night, and 4.12 ± 0.02 h on the third night ( $F(1,12) = 3495$ , *p* < 0.001 for the main effect of *Sleep condition*).

### 3.2. Daytime variations of plasma concentrations of endocannabinoids

When considering all time points collected in the present study (i.e., day 3: 7:30 p.m., day 4: 8:30 a.m., 10 a.m., as well as 15 min (~11 a.m.) and 4 h post-exercise (~3 p.m.)) across both sleep conditions, repeated measures ANOVA revealed a strong main effect of time on plasma concentrations of 2AG (*p* < 0.001, lowest concentration at 10 a.m. on day 4, 2.04 ± 0.16 pmol/ml; highest concentration at 8:30 a.m. on day 4, 3.10 ± 0.44 pmol/ml), AEA (*p* < 0.001, lowest concentration at 8:30 a.m. on day 4, 0.82 ± 0.04 pmol/ml; highest concentration at 7:30 p.m. on day 3, 1.16 ± 0.06 pmol/ml), PEA (*p* < 0.001, lowest concentration at 8:30 a.m. on day 4, 14.48 ± 0.53 pmol/ml; highest concentration at ~3 p.m. on day 4, 18.58 ± 0.68 pmol/ml), and OEA (*p* < 0.001, lowest concentration at 8:30 a.m. on day 4, 5.18 ± 0.26 pmol/ml; highest concentration at ~3 p.m. on day 4, 7.42 ± 0.33 pmol/ml).

### 3.3. Effects of nocturnal sleep duration on plasma concentrations of endocannabinoids

A detailed summary of repeated measures ANOVA main and interaction effects of within-subject factors *Sleep condition* and *Pre/Post-sleep*, or *Exercise time point*, on plasma endocannabinoid concentrations is shown in Table 1.

#### 3.3.1. Plasma concentrations of endocannabinoids before exercise

In the morning of day 4 (i.e., at 8:30 a.m. following 3 experimental nights), plasma concentrations of 2AG were on average +80% higher in the short vs. normal sleep condition (3.99 ± 0.74 vs. 2.22 ± 0.21 pmol/ml, *df* = 15, *t* = 2.78, *p* = 0.014; Fig. 2A). No differences were, however, observed in the evening of day 3 (i.e., following 2 experimental nights) between sleep conditions (*p* = 0.830, pairwise *t*-test comparison; Fig. 2A).

Plasma concentrations of AEA, PEA, and OEA did not differ between the sleep conditions (*p* ≥ 0.937 for all main effects of *Sleep condition*; *p* ≥ 0.423 for all interaction effects of *Sleep condition* with *Pre/Post-sleep*; Table 1 & Fig. 2B–D). In contrast, significant main effects of *Pre/Post-sleep* on plasma concentrations of AEA and PEA were found, as morning values of day 4 were lower than those measured in the evening before (Table 1 & Fig. 2B,C). No such main effects of *Pre/Post-sleep* were, however, observed for plasma concentrations of OEA (*p* = 0.263, Table 1 & Fig. 2D).

#### 3.3.2. Effects of sleep duration on plasma concentrations of endocannabinoids following exercise

As summarized in Table 1, repeated measures ANOVA revealed neither main effects of *Sleep condition* nor interaction effects of *Sleep condition* with *Exercise time point* on plasma concentrations of 2AG, AEA, PEA, and OEA (see also Fig. 3A–D). In contrast, main effects of *Exercise time point* on plasma concentrations of 2AG and OEA were found (*p* ≤ 0.036 for all main effects of *Exercise time point*; Table 1). Post-hoc comparisons

**Table 1**  
Repeated measures ANOVA main and interaction effects on plasma endocannabinoid concentrations across sleep conditions and before and after exercise.

EC	Within-subject factors				Interaction	
	<i>Before exercise</i> <i>Sleep condition</i>	<i>P</i>	<i>Pre/Post-sleep</i>	<i>P</i>	<i>Sleep condition * Pre/Post-sleep</i>	<i>P</i>
2AG	F(1.0,15.0)=4.12	0.060	F(1,15)=0.27	0.610	F(1.0,15.0)=7.56	0.015
AEA	F(1.0,15.0)=0.0004	0.984	F(1,15)=31.91	0.00005	F(1.0,15.0)=0.68	0.423
PEA	F(1.0,15.0)=0.006	0.941	F(1,15)=6.77	0.020	F(1.0,15.0)=0.06	0.810
OEA	F(1.0,15.0)=0.007	0.937	F(1,15)=1.35	0.263	F(1.0,15.0)=0.009	0.924

EC	Within-subject factors				Interaction	
	<i>Following exercise</i> <i>Sleep condition</i>	<i>P</i>	<i>Exercise time point</i>	<i>P</i>	<i>Sleep condition * Exercise time point</i>	<i>P</i>
2AG	F(1.0,15.0)=0.55	0.472	F(1.22,18.36)=8.05	0.008	F(1.28,19.19)=7.56	0.280
AEA	F(1.0,15.0)=0.47	0.502	F(1.80,27.06)=31.91	0.332	F(1.54,23.13)=0.68	0.406
PEA	F(1.0,15.0)=0.60	0.452	F(1.59,23.91)=3.45	0.058	F(1.63,24.47)=2.05	0.157
OEA	F(1.0,15.0)=0.28	0.605	F(1.48,22.27)=4.30	0.036	F(1.49,22.32)=1.43	0.256

Repeated measures ANOVA was conducted to investigate the influence of recurrent short sleep duration on fasting circulating concentrations of endocannabinoids (within-subject factors: *Sleep condition* and *Pre/Post-sleep* → day 3: 7:30 p.m., day 4: 8:30 a.m.) and whether recurrent short sleep duration would alter the effects of acute physical exercise on circulating concentrations of endocannabinoids (within-subject factors: *Sleep condition* and *Exercise time point* → day 4: ~10 a.m. (last pre-exercise time point), as well as 15 min and 4 h post-exercise). The Greenhouse-Geisser method was used to correct for sphericity deviations. *Abbreviations*: 2AG, 2-arachidonoylglycerol; AEA, anandamide; EC, endocannabinoid; PEA, palmitoylethanolamide; OEA, oleoylethanolamide.

revealed that plasma concentrations of 2AG were only significantly different when pre-exercise and 15 min post-exercise time points were compared (15 min post-exercise vs. pre-exercise:  $2.94 \pm 0.40$  vs.  $2.04 \pm 0.16$  pmol/ml,  $p=0.012$ ; +4 h post-exercise,  $2.25 \pm 0.22$  pmol/ml vs. pre-exercise:  $p=0.196$ ; 4 h post-exercise vs. 15 min post-exercise:  $p=0.093$ ; all Bonferroni-corrected; Fig. 3A). Finally, plasma concentrations of OEA differed only between pre-exercise and 4 h post-exercise time points (15 min post-exercise vs. pre-exercise:  $6.77 \pm 0.45$  vs.  $6.29 \pm 0.33$  pmol/ml,  $p=0.896$ ; 4 h post-exercise,  $7.42 \pm 0.33$  pmol/ml, vs. pre-exercise:  $p=0.001$ ; 4 h post-exercise vs. 15 min post-exercise:  $p=0.466$ ; all Bonferroni-corrected; Fig. 3D).

There were no significant main effects of *Exercise time point* on plasma concentrations of AEA and PEA ( $p \geq 0.058$ ; Table 1 & Fig. 3B,C).

### 3.4. Effects of sleep duration on hunger and stress ratings

In the morning of day 4 following either 3 nights of short or normal sleep duration, participants felt hungrier at 8:30 a.m. in the short sleep condition than they did in the normal sleep condition (+25%,  $p=0.034$ ). No other differences in hunger feelings were observed on day 4 between the sleep duration conditions ( $F(2.23,33.22)=2.55$ ,  $p=0.088$  for *Sleep condition\*Time*;  $F(1.0,15.0)=0.04$ ,  $p=0.843$  for *Sleep condition*;  $F(2.74,41.08)=14.94$ ,  $p<0.001$  for *Time*; Fig. 4A).

Concerning stress, participants in general felt more stressed in the normal sleep condition than they did in the short sleep condition ( $F(2.26,33.95)=0.95$ ,  $p=0.407$  for *Sleep condition\*Time*;  $F(1.0,15.0)=8.57$ ,  $p=0.010$  for *Sleep condition*;  $F(2.47,37.03)=2.83$ ,  $p=0.061$  for *Time*), especially for ratings done after the physical exercise task had been completed (i.e., 15 min post-exercise and 4 h post-exercise; Fig. 4B).

When considering individual ratios (i.e., individual values at each time points on day 4 in the short sleep condition were divided by corresponding time-matched individual values in the normal sleep condition, e.g. plasma concentration of 2AG at 8:30 a.m. in the short sleep condition was divided by plasma concentration of 2AG at 8:30 a.m. in the normal sleep condition), no correlations were found of subjective hunger and stress ratings with plasma endocannabinoid concentrations (Pearson's  $r$  (min, max): 2AG/AEA/PEA/OEA vs. hunger,  $-0.072$ ,  $0.027$ ,  $p \geq 0.570$ ; vs. stress,  $-0.042$ ,  $0.159$ ,  $p \geq 0.210$ ).

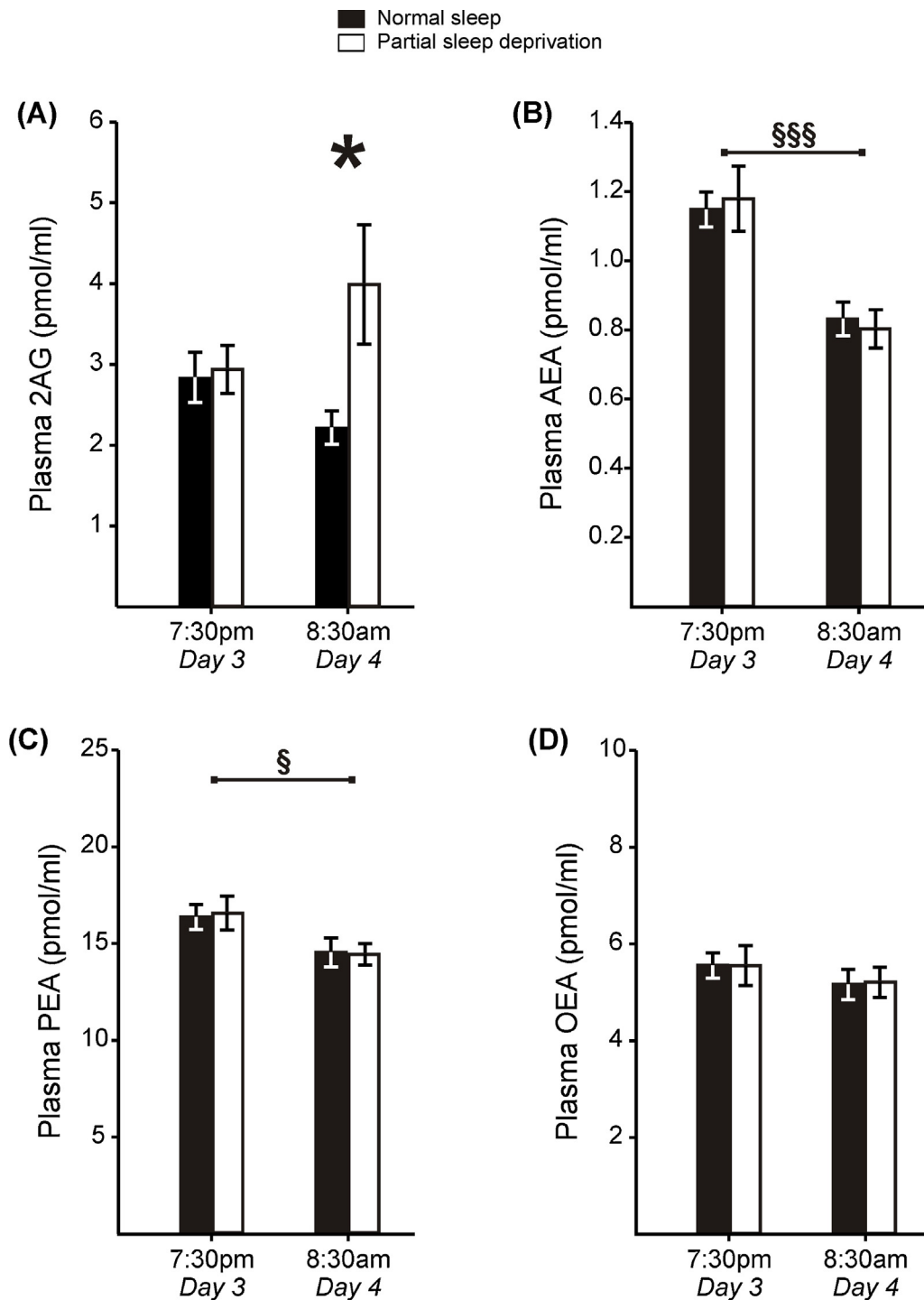
## 4. Discussion

In this study involving 16 healthy young men, we aimed to investigate if recurrent short sleep duration over three consecutive nights would alter systemic concentrations of endocannabinoids, both prior to and following 30 min of high-intensity ergometer cycling.

### 4.1. Comparison with the sleep literature

#### 4.1.1. Effects of sleep restriction on plasma 1/2AG levels

Our study found a rise in plasma 2AG concentrations shortly after awakening after three nights of short sleep (sleep window 2:45–7 a.m.) vs. normal sleep (sleep window 22:30 p.m.–7 a.m.). This is in contrast to results from a recent study involving 14 healthy young adults in which circulating concentrations of 2AG were found to be primarily increased during afternoon hours (around 3 p.m.) following four nights of short sleep (sleep window 1–5:30 a.m.) vs. four nights of normal sleep (sleep window 11 p.m.–7:30 a.m.) (Hanlon et al., 2016). Another finding in our study was that plasma 2AG concentrations in the afternoon (around 3 p.m.) returned to pre-exercise values, an effect that was seen in both the short and normal sleep condition. This also differs from recent results demonstrating that plasma 2AG exhibits a distinct diurnal pattern across a normal sleep-wake cycle, peaking in early-mid-afternoon (Hanlon et al., 2015). Differences in meal timing, type of food, duration of fasting, and gender distribution could offer possible explanations for discrepant results across experiments, since all of these factors have been shown to alter endocannabinoid production (Gatta-Cherifi et al., 2012; Engeli et al., 2014; Kirkham et al., 2002; Blüher et al., 2006; Di Marzo et al., 2001). Additionally, differences in time awake with light exposure in the morning (2-h difference in the study by Hanlon et al. (2016) compared with equal time across our study conditions), as well as differences in sleep midpoints between the sleep conditions (3:15 a.m. across both sleep conditions in Hanlon et al., 2016; 2:45 a.m. and 4:53 a.m. in our normal and partial sleep restriction conditions herein, respectively) may explain the differing results observed across these studies. Finally, physical exercise has been demonstrated to be able to shift circadian rhythms of both central and peripheral tissues (Schroeder et al., 2012; Wolff and Esser, 2012). With this in mind, the morning exercise bout performed in the present study could be the rea-



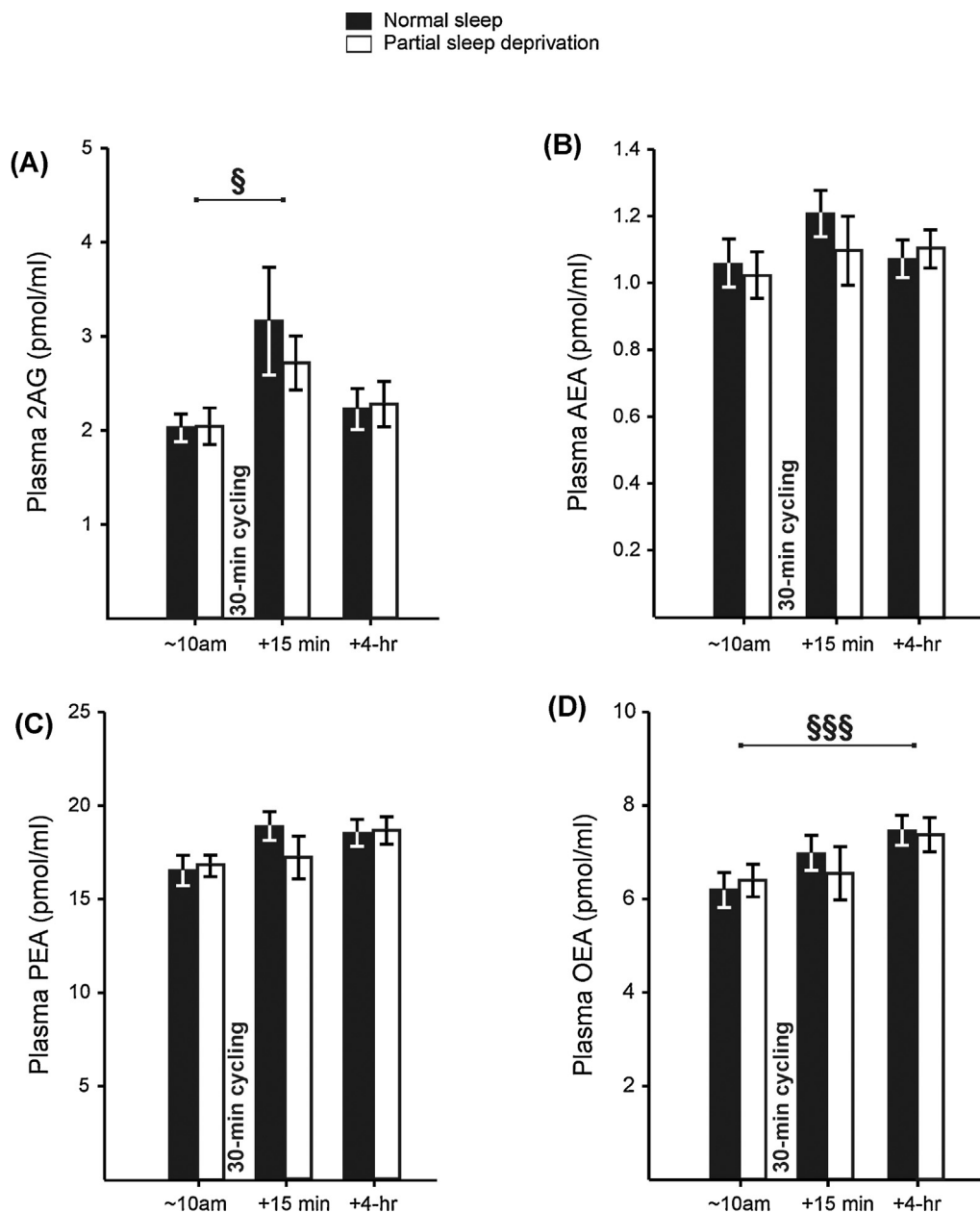
**Fig. 2.** Overnight effects of three nights of either short or normal sleep on plasma concentrations of (A) 2-arachidonoylglycerol (2AG), (B) anandamide (AEA), (C) palmitoylethanolamide (PEA), and (D) oleoylethanolamide (OEA) before exercise. Analyzed data points are from blood samples taken at 7:30 p.m. on day 3 and at 8:30 a.m. on day 4. \*,  $p < 0.05$  for NS vs. PSD. §,  $p < 0.05$ ; and §§§,  $p < 0.001$  for Bonferroni-corrected time point comparisons.

son for why plasma 2AG did not peak in the afternoon and did not differ between the short and normal sleep conditions at 3 p.m.

The increase we observed in plasma 2AG due to sleep restriction was transient, as it occurred shortly after awakening at 8:30 a.m., but was no longer apparent at 10 a.m. on day 4. Given that endocannabinoids are responsive to glucocorticoid hormones (Hill et al., 2010b) and mediate many of the physiological actions of these hormones in both central and peripheral tissues (Bitencourt et al.,

2014; Bowles et al., 2015; Hill et al., 2011), an elevated cortisol awakening response (CAR) following partial sleep loss could represent a mechanism underlying the transient increase in 2AG at 8:30 a.m. on day 4. Supporting this view, a recent study involving 2751 participants found short sleep duration to be associated with an increased CAR (Kumari et al., 2009).

Contrary to 8:30 a.m., no differences in plasma 2AG concentrations were seen at 10 a.m. (i.e., 3 h after awakening) on day 4 between the sleep conditions. One explanation could be partici-



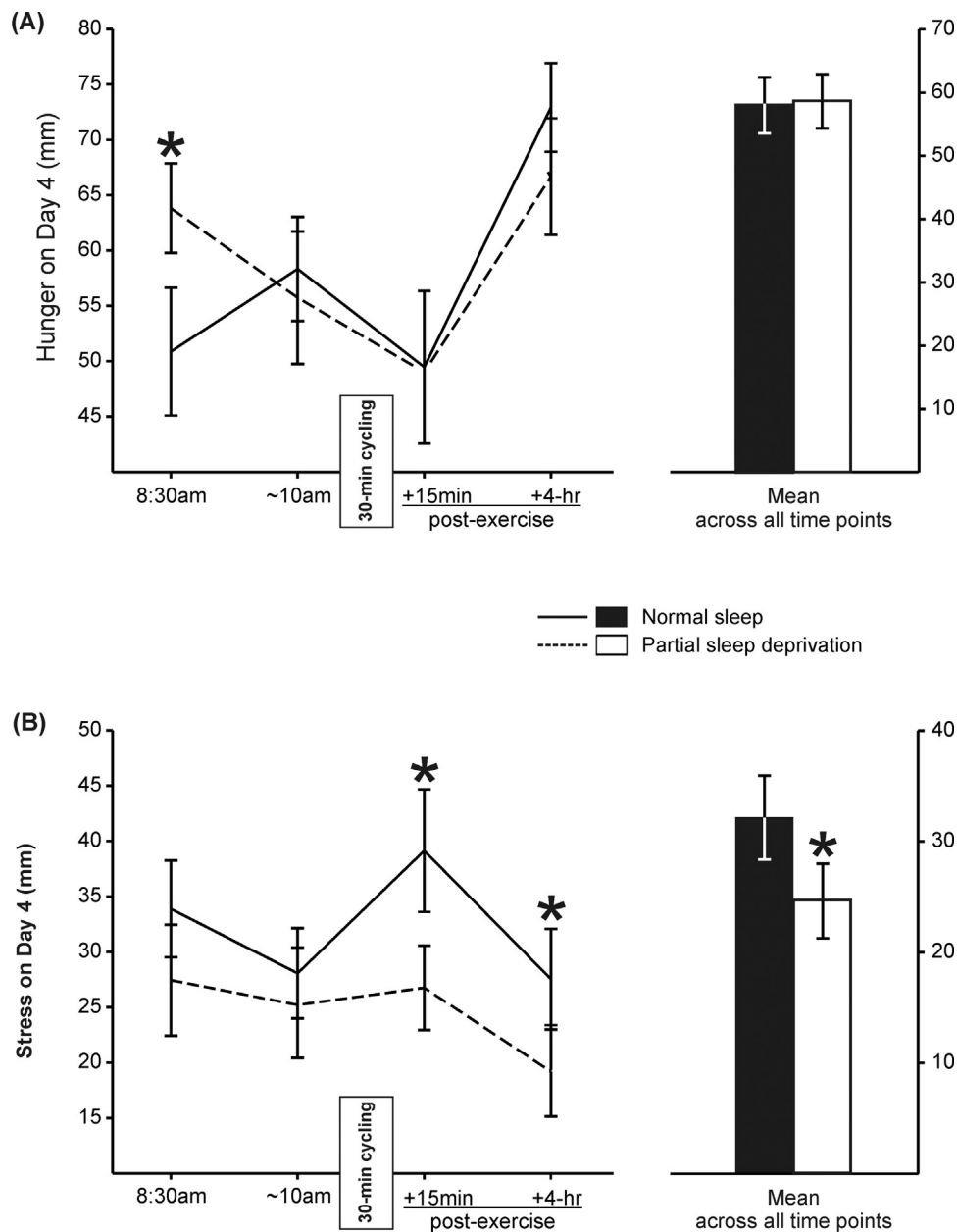
**Fig. 3.** Effects of an acute bout of physical exercise on plasma concentrations of (A) 2-arachidonoylglycerol (2AG), (B) anandamide (AEA), (C) palmitoylethanolamide (PEA), and (D) oleoylethanolamide (OEA) following three nights of either short (4.25-h) or normal (8.5-h) sleep duration. Analyzed data are from blood samples collected before exercise (~10 a.m.), as well as 15 min and 4 h after the end of the exercise protocol. §,  $p < 0.05$  and §§§,  $p < 0.001$  for Bonferroni-corrected time point comparisons; points connected along the horizontal lines below the significance symbols indicate the time points that are significantly different to one another.

pants' cumulative bright light exposure with increasing elapsed wakefulness. Light is known to alter the activity of biological clocks regulating energy and lipid metabolism (Bass and Takahashi, 2010). Thus, it could be hypothesized that possible effects of recurrent partial sleep loss on plasma endocannabinoid concentrations were no longer modulated by CAR at 10 a.m., but by then mostly driven by underlying circadian rhythms.

#### 4.1.2. Effects of sleep restriction on plasma AEA, PEA, and OEA levels

Plasma concentrations of AEA (and its congeners PEA, and OEA) did not differ between the short and normal sleep duration conditions, which is somehow at odds with previous findings. For instance, in a study involving five healthy humans, circulating

AEA concentrations increased from nighttime to morning and then declined again closer to nighttime when participants were kept under a normal sleep-wake cycle. In contrast, this pattern was disrupted following one night of total sleep deprivation (e.g. concentrations of plasma AEA did not decline over the day), indicating that the absence of sleep produces a significant dysregulation of circulating AEA (Vaughn et al., 2010). However, others have reported that total sleep loss did not alter circulating AEA concentrations (Koethe et al., 2009), whereas they instead found increased levels of OEA in CSF but not in plasma (Koethe et al., 2009). The latter finding is of particular importance for the interpretation of our null results, as it suggests that the extent by which sleep loss alters endocannabinoid signaling may vary across tissues and/or sampled biological fluids.



**Fig. 4.** (A) Hunger and (B) stress ratings on the fourth day, following three nights of either short sleep or normal sleep duration. \* $p < 0.05$  for normal sleep vs. partial sleep deprivation.

**4.2. Effects of exercise on plasma endocannabinoid concentrations**

We found that exercise carried out in the fasted state induced a short-term rise (15-min into the recovery period post exercise) in plasma concentrations of 2AG, whereas no statistically significant effects on plasma AEA concentrations were found. Similar effects have been found after acute psychological stress (Hill et al., 2009). Studies utilizing acute physical exercise as stressor have however found different responses, *i.e.*, an increase of plasma concentrations of AEA but not 2AG (Heyman et al., 2012; Raichlen et al., 2013; Sparling et al., 2003). Differences in exercise intensity, circadian timing of exercise, timing of blood sampling (*i.e.*, during or different time points after exercise), and pre-exercise conditions (*e.g.* photic preload, time since last meal, macronutrient composition of last meal, and gender distribution) could offer possible explanations for why we observed an increase in plasma concentrations

of 2AG but not AEA, whereas previous studies found exercise to increase plasma concentrations of AEA but not 2AG (Heyman et al., 2012; Raichlen et al., 2013; Sparling et al., 2003). Another consideration when interpreting our data is that a previous study found plasma 2AG concentrations to increase by around 17% under resting conditions from 10 a.m. to 11 a.m. (Hanlon et al., 2015), *i.e.*, corresponding to the time period during which we found exercise to elevate plasma concentrations of 2AG. While we observed an increase much greater (about 44%) than this observed diurnal fluctuation, the elevated 2AG levels after ergometer cycling may nevertheless partially have been due to the concurrent natural rise along its diurnal pattern in blood.

An additional finding of the present study is that the exercise-induced rise in plasma concentrations of 2AG did not differ between sleep conditions. The increase of circulating levels of endocannabinoids following exercise has been proposed to partially account for the anxiolytic (Tantimonaco et al., 2014), anti-nociceptive (Galdino



et al., 2014) and neuroprotective (Hill et al., 2010a) properties of physical exercise. Indeed, recent research suggests that at least in mice, central and peripheral CB1 and CB2 receptors are necessary for inducing anxiolysis and analgesia after running (Fuss et al., 2015). Our results could therefore indirectly suggest that recurrent partial sleep loss does not alter these centrally acting benefits of exercise. Importantly however, sleep deprivation may have modulated post-exercise plasma concentrations of endocannabinoids before, in between, or after, the time points measured in our study, i.e., 15 min and 4 h after exercise. In light of our findings that partial sleep loss increased pre-exercise plasma concentrations of 2AG only at 8:30 a.m. but not at 10 a.m., differences in plasma endocannabinoid concentrations between sleep conditions might have occurred if exercise would have been scheduled closer to awakening.

Finally, an increase of plasma concentrations of OEA (AEA congener) was found at 4 h post-exercise. However, since this rise in plasma OEA occurred relatively late after physical exercise, this could also represent a circadian fluctuation, rather than an exercise effect – the circadian profile of this endocannabinoid has however not yet been properly defined in humans. Since OEA has been shown to activate peroxisome proliferator-activated receptor alpha (PPAR-alpha) (Guzmán et al., 2004), a major regulator of lipid metabolism that is activated to induce a metabolic adaptive response to prolonged fasting (Kersten et al., 1999), the rise of this lipid metabolite in plasma could also stem from extended energy deprivation, exacerbated by the exercise intervention.

#### 4.3. Sleep loss increases hunger feelings: possible role of 2AG

A well-known effect of cannabinoids is increased food craving and calorie intake (Kirkham, 2009). For instance, intraperitoneal administration of rimonabant (an antagonist for the cannabinoid receptor CB<sub>1</sub>) reduces food intake (Thornton-Jones et al., 2006) and induces neuronal activation in several hypothalamic nuclei involved in food intake regulation, such as the paraventricular nucleus (Verty et al., 2009). Furthermore, administration of 2AG into the shell of the nucleus accumbens, an extra-hypothalamic area strongly linked to eating motivation increases food intake (Kirkham et al., 2002). With these findings in mind, it could therefore be hypothesized that the rise in plasma 2AG (and the sum with its isomer 1AG) may offer a possible molecular mechanism for findings of previous studies, which have demonstrated that sleep restriction increases craving and intake of food during morning hours (Benedict et al., 2012; Cedernaes et al., 2014; Chapman et al., 2013; Hogenkamp et al., 2013; St-Onge et al., 2011), and possibly also a reason for the previously observed elevated risk of long term weight gain and obesity in those who chronically suffer from insufficient sleep (Cappuccio et al., 2008). However, three nights of partial sleep loss increased plasma concentrations of 2AG only shortly after awakening in the present study (i.e., 8:30 a.m.), which is therefore not likely to represent the main mechanism that promotes overeating across 24-h in sleep-deprived people (St-Onge et al., 2011) – unless it is able to produce a more long-lasting effect at the behavioral or energy homeostasis level. Supporting the notion of a more short-term effect of elevated endocannabinoids, no correlations were found between plasma endocannabinoid concentrations and subjective hunger ratings.

#### 4.4. Acute sleep loss reduces subjective stress: possible role of 2AG

An additional observation of our study is that participants generally felt less stressed following three nights of short sleep vs. normal sleep, particularly following the physical exercise protocol. While chronic sleep loss has been associated with increased psychological stress (Akerstedt, 2006), acute sleep deprivation –

foremost total sleep deprivation – has been shown to exert transient mood-improving effects (Wu et al., 1999). Given that low doses of CB1 agonists tend to be anxiolytic and high doses tend to increase aversion and anxiety-related behaviors (Ganon-Elazar and Akirav, 2012; also reviewed in Viveros et al., 2005), it could be that the small but significant increase in plasma concentrations of 2AG shortly after awakening may have mediated the subjective stress-reducing effects of short sleep duration. It must however be kept in mind that we did not find any correlations between subjective stress and plasma concentrations of endocannabinoids. Moreover, caution is warranted before concluding based on our findings that humans can cope better with stressors under conditions of acute sleep loss. For instance, there is some evidence that humans are more responsive to the effects of psychological stress when sleep-deprived compared to when they are well-rested (Cedernaes et al., 2015b; Minkol et al., 2014). Finally, sleep restriction has also been shown to promote adverse psychological symptoms (Roy-Byrne et al., 1986).

## 5. Conclusions

Given that activation of the endocannabinoid system has been previously shown to increase acutely appetite and mood (Di Marzo et al., 2001; Lutz et al., 2015), our results could suggest that behavioral effects of acute sleep loss, such as increased hunger and transiently improved psychological state, may partially result from activation of this signaling pathway. In contrast, exercise-induced elevations of endocannabinoids appear to be less affected by short sleep duration. Whether our findings are generalizable to females, older participants, or obese humans living under short sleep conditions, or whether similar results would be seen if exercise would have been carried out following an energy preload, is currently unknown and would be worth investigating.

## Conflict of interest

The authors are unaware of any affiliation, funding, or financial holdings that might be perceived as affecting the objectivity of this manuscript. The authors declare that there is no biomedical financial interest or potential conflict of interest.

## Role of the funding sources

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Contributors

JC and CB designed the study; JC and CB wrote the protocol; JC, and JEB collected the data; JC, FF, AF and CB conducted the analyses. All authors interpreted the data; and all authors contributed to writing. All authors have approved the final manuscript.

JC and CB had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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