Title:

Cerebral A_1 adenosine receptor availability in female and male participants and its relationship to sleep

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Abstract

The neuromodulator adenosine and its receptors are mediators of sleep-wake regulation which is known to differ between sexes. We, therefore, investigated sex differences in A_1 adenosine receptor (A_1AR) availability in healthy human subjects under well-rested conditions using [^{18}F]CPFPX and positron emission tomography (PET). [^{18}F]CPFPX PET scans were acquired in 50 healthy human participants (20 females; mean age ± SD 28.0 ± 5.3 years). Mean binding potential (BP_{ND}; Logan's reference tissue model with cerebellum as reference region) and volume of distribution (V_T) values were calculated in 12 and 15 grey matter brain regions, respectively. [^{18}F]CPFPX BP_{ND} was higher in females compared to males in all investigated brain regions ($p < 0.025$). The largest differences were found in the pallidum and anterior cingulate cortex, where mean BP_{ND} values were higher by 29 % in females than in males. In females, sleep efficiency correlated positively and sleep latency negatively with BP_{ND} in most brain regions. V_T values did not differ between sexes. Sleep efficiency correlated positively with V_T in most brain regions in female participants. In conclusion, our analysis gives a first indication for potential sex differences in A_1AR availability even under well-rested conditions. A_1AR availability as measured by [^{18}F]CPFPX BP_{ND} is higher in females compared to males. Considering the involvement of adenosine in sleep-wake control, this finding might partially explain the known sex differences in sleep efficiency and sleep latency.

Keywords:

Adenosine receptors, A_1AR, PET, sex differences, [^{18}F]CPFPX, sleep

1. Introduction

Sex differences in brain function, structure, and chemistry gained interest in recent years (Cosgrove et al., 2007). Sex differences have been described in several neurotransmission systems, including the serotonin (5-HT), dopamine, acetylcholine, and metabotropic glutamate receptor systems. For instance, higher serotonin 5-HT_{1A} receptor (Jovanovic et al., 2008), 5-HT transporter (5-HTT) (Staley et al., 2001), dopamine D_2 receptor (Kaasinen et al., 2001), and muscarinic acetylcholine receptor (Yoshida et al., 2000) availability were consistently found in females when compared to males. Such sex-related differences in the neurochemical architecture of the brain may contribute to sex-specific phenotypes of brain diseases and necessitate an adjustment of current standard treatments.

Here we asked whether sex differences are also present in the cerebral adenosine receptor system. So far, this question has not been studied extensively. Mostly, the presence or absence of such differences were reported as secondary observations, but they were not the primary focus of the dedicated studies. Thus, it is not surprising that the available results are inconsistent. In a recently published positron emission tomography (PET) study, higher A_1 adenosine receptor (A_1AR) availability was found in females compared to males, specifically in the amygdala and thalamus (personal correspondence with the authors), when examining confounding effects (Hohoff et al., 2020). In contrast, no sex-specific differences in A_1AR binding were found in autoradiography studies on post-mortem brain slices (Glass et al., 1996; Ułas et al., 1993).

The adenosine receptor system consists of four G-protein coupled receptor types, A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR, of which A_1AR is most widely distributed in the brain (for review see (Ribeiro et al., 2002)). The A_1AR has been suggested to be involved in the modulation of the sleep-wake cycle and the regulation of polysomnographic (PSG [*]) markers of sleep intensity and sleep need such as the duration of deep slow-wave sleep (SWS) as well as delta frequency activity in non-rapid-eye-movement (NREM) sleep (for review see (Basheer et al., 2004; Huang et al., 2011; Lazarus et al., 2019)). However, sleep-wake behaviour also affects A_1AR availability. It was shown that prolonged wakefulness, and consequently increased sleep pressure, resulted in increased A_1AR mRNA levels and receptor density in rats (Basheer et al., 2001; Elmenhorst et al., 2009) and A_1AR availability in humans (Elmenhorst et al., 2017; Elmenhorst et al., 2007b).

[*]**Abbrevitions:** PSG, polysomnographic; SWS, slow-wave sleep; NREM, non-rapid-eye-movement; TIB, time-in-bed; TLC, thin-layer chromatography; SPT, sleep period time; REM, rapid-eye-movement; N1, NREM sleep stage 1; N2, NREM sleep stage 2; N3, NREM sleep stage 3; %SPT, percentage of SPT; SWA, slow-wave activity

Just as brain structure and chemistry are subject to sex differences, sleep behaviour also depends on sex. For example, earlier sleep onset and wake onset as well as elevated NREM sleep delta activity were consistently found in females when compared to males (Carrier et al., 2001; Dijk et al., 1989; Valomon et al., 2014). Given the sex-specific differences in sleep behaviour and the role of A_1AR in sleep-wake regulation, we expected to find sex-related differences in A_1AR availability. Therefore, we used [^{18}F]CPFPX PET to investigate A_1AR availability in terms of the [^{18}F]CPFPX binding potential (BP_{ND}) and the total distribution volume (V_T) in the human brain under PSG-confirmed, well-rested conditions. The results may contribute to a better understanding of the potential mechanisms underlying the effects of sex on sleep behaviour and quality.

2. Methods

2.1. Participants

Fifty healthy human volunteers (20 females and 30 males; mean age ± SD 28.0 ± 5.3, range 21 – 39 years) from two studies (see below) were included in the present analysis. All procedures were approved by the Ethics Committee of the regional Medical Board (Ärztekammer Nordrhein) and the German Federal Office for Radiation Protection. Each participant gave written, informed consent. Exclusion criteria were as follows: chronic neurological or psychiatric disorders, head trauma, sleep disorder, shift or night work, alcohol and drug abuse, smoking, pregnant or breast-feeding females. Only participants reporting no current medication (except contraceptives) and an estimated habitual caffeine consumption below 450 mg/day were included in the present investigation. Participants had to abstain from alcohol and caffeine one week before arriving at the sleep lab and during their time in the lab before the PET scan. On the day of arrival at the lab, participants' urine was tested for the following substances: cotinine, zolpidem, propoxyphene, amphetamines, barbiturates, benzodiazepines, cocaine, methamphetamines, heroin morphine, methadone, ecstasy, tricyclic antidepressants, tetrahydrocannabinol. The volunteers participated in one of two studies with slightly different study designs (for full experimental design of study #1 (15 controls, 5 females) and #2 (35 participants from both groups, 15 females) see (Hennecke et al., 2020) and (Baur et al., 2020), respectively). Participants of study #1 were not pre-selected for a specific genotype, but with exception of two participants, all gave written consent for later genotyping. Most participants of study #2 were pre-selected for the homozygous C/C variant of the rs5751876 allele. In total, 14 females and 17 males were C/C homozygous, two males were T/T homozygous, six females and nine males were C/T heterozygous, and two males were not genotyped (Table 1).

2.2. Sleep studies

Before arriving at the sleep research lab, all participants reported their habitual sleep behaviour on working days and followed a one-week ambulatory sleep satiation protocol (9 hours time-in-bed (TIB); 10:00/11:00 p.m. – 07:00/08:00 a.m.), which was verified by actometer recording and sleep diaries. In the lab, the scheduled sleep episode was 8 hours in duration (TIB; 11:00 p.m./12:00 a.m. – 07:00/08:00 a.m.) and sleep data were recorded using PSG as described in (Hennecke et al., 2020). The illuminance was ~100 lx. Participants had to abstain from caffeine and alcohol during the preparatory week at home and during the time in the sleep lab. Participants of both studies spent one adaptation night and two baseline nights (8 hours TIB) in the sleep lab, thereafter volunteers of study #1 were scheduled to sleep for 8 hours (TIB) for five consecutive days in the lab and were scanned the following day (i.e., after 8 nights in the lab), whereas volunteers of study #2 were scanned after the second baseline night (i.e., after 3 nights in the lab).

2.3. [^{18}F]CPFPX PET data acquisition

[^{18}F]CPFPX formulation and synthesis were performed as previously described (Holschbach et al., 2002). Chemical purity was always above 96 %. The radioligand was diluted with sterile saline solution (0.9 %) and administered using a standard syringe pump. The radiotracer was injected as an intravenous bolus (15.9 ml in 2 min) followed by constant infusion (34.1 ml in 118 min) with a K_{bol} value of 55 min (Elmenhorst et al., 2007a). Scan duration was 100 min. The mean injected dose of [^{18}F]CPFPX was 175.9 ± 21.8 MBq (range 103 – 200 MBq), molar activity at injection time was 102.25 ± 72.08 GBq/µmol (range 19.24 - 317.62 GBq/µmol). The corresponding mass of injected CPFPX was 2.70 ± 1.89 nmol on average (0.37 – 9.61 nmol). Injection and scan were started simultaneously at 13:54:23 ± 01:49:40 on average (range 11:02:52 – 17:41:33).

[^{18}F]CPFPX PET data acquisition and high-resolution three-dimensional T1-weighted magnetic resonance (MR) imaging were conducted on an integrated 3 Tesla whole-body PET/MR system (Biograph mMR, Siemens Healthineers) (Delso et al., 2011) at the German Aerospace Centre (Cologne). The PET scanner was calibrated on a daily basis and normalised using a ^{68}Ge/^{68}Ga phantom. An aliquot of the ^{68}Ge/^{68}Ga phantom was counted in a γ-counter (Wizard²; PerkinElmer) to determine cross-calibration factor. PET data were acquired in list mode. Reconstruction was done with e7 tools (Siemens Molecular Imaging) using OP-OSEM reconstruction algorithm with point spread function modelling with 3 iterations and 21 subsets. A 3 mm Gaussian filter for post-filtering was used. The framing scheme was 4 x 60 s, 3 x 120 s, 18 x 300 s. The resulting PET images have matrix dimensions of 344 x 344 x 127 with a reconstructed image resolution of 2.09 x 2.09 x 2.03 mm³. They were corrected for detector normalisation, randoms and scatter. Template based attenuation correction was based on the method described in (Izquierdo-Garcia et al., 2014).

Arterialised venous blood samples were manually drawn at 2, 50, 60, 70, 80, 90, and 100 min after the start of [^{18}F]CPFPX infusion. During the equilibrium phase of [^{18}F]CPFPX bolus/infusion experiments, venous and arterial concentrations equilibrate, consequently venous blood sampling can substitute arterial withdrawals (Elmenhorst et al., 2007b; Meyer et al., 2005). All blood samples were collectively analysed immediately after the PET scan was completed. Whole blood samples (500 µl) were counted in cross-calibrated γ-counter for 120 s. Blood samples were centrifuged (3000 g, 3 min) to obtain plasma. Plasma samples (400 µl) were mixed with extraction solution (acetonitrile / methanol 50/50 v/v, 400 µl), vortexed for 60 s at room temperature, counted in the γ-counter in duplicates, and then centrifuged at 18 °C (20,000 g, 2 min). Aliquots (3 x 5 µl) of supernatants were applied to a pre-coated thin-layer chromatography (TLC) plate (809022; Macherey-Nagel) and developed with a mobile phase of ethyl acetate / heptane 75/25 (v/v) to analyse unmetabolized [^{18}F]CPFPX. The pellets were measured in a γ-counter in duplicates. TLC plates were exposed to imaging plates type HCR (HR2025cm113; Dürr NDT) for 3-5 h. Imaging plates were scanned using an image plate reader (CR 35 Bio Plus; Dürr Medical) and analysed with AIDA Imaging Analysis software (Elysia Raytest). Whole blood, plasma, and pellet radioactivity were decay-corrected to scan start. The time courses of the fraction of the total radioactivity extraction relative to the 2-min sample and the fraction of parent compound in the plasma were fitted by non-linear regression analyses (Elmenhorst et al., 2007a; Meyer et al., 2004; Meyer et al., 2005). These fits were used to generate metabolite and extraction-corrected plasma input functions.

2.4. Data analysis

Pre-processing of PET and corresponding MRI data were done with PMOD Neuro Tool (version 4.006; PMOD Technologies). PET data were motion corrected to a reference image, which was created by averaging PET data of the first 9 min of the scan. Matching parameters were kept at default, including squared difference sum cost function, trilinear interpolation, and smoothing using 6 mm full width at half maximum (FWHM). In case automatic segmentation failed, MR images were cropped using the automatic cropping function. This removed the neck and limited the MR data set to skull and brain. T_1-weighted MR images were segmented into grey matter (GM), white matter (WM), and cerebrospinal fluid. For this, denoising of the MR images was done at medium strength, followed by segmentation using the 6 Probability Maps (SPM12) variant. The sampling parameter was set to 3.0 mm, bias regularisation compensated for light modulations of the image intensity across the field-of-view and variations were smoothed using 60 mm FWHM. Clean-up setting was set to thorough and affine regularization was initialised according to European brains. Segmentation touch-up was done using a background 0.2 probability level and the overlay strategy with thresholds for the GM and the WM probability

map of 0.1 and 0.05, respectively. In case PET-MR matching was required, rigid matching based on the normalised mutual information criterion with matching sampling of 3.0 pixel was applied. Spatial normalisation was performed using probability maps transformation, which uses the normalisation results from the previous MR segmentation. 70 volumes of interests (VOIs) were defined by the automated anatomical labelling template in the Montreal Neurological Institute space implemented in the PMOD software (Tzourio-Mazoyer et al., 2002). The PET images were evaluated in atlas space and GM probability information was applied from the segmentation resulting mask. Borders of cortical VOIs were checked and manually adjusted to avoid misdetection of signal from cerebral sinuses. The cerebellar VOIs were manually adapted to enable the usage of the cerebellum as a reference region, which is defined by a low A_1AR availability (Bauer et al., 2003; Fastbom et al., 1987; Meyer et al., 2007).

Kinetic modelling was done with PMOD Kinetics Tool (version 4.006; PMOD Technologies). Regional time-activity curves (TACs) were calculated for each VOI. TACs of the left and right cerebellum were averaged to define the new TAC of the reference region. Decay-corrected whole blood function and decay, metabolite and extraction-corrected plasma input function were used to correct regional TACs. Corrected TACs were used to estimate the A_1AR availability in terms of the [^{18}F]CPFPX BP_{ND} and V_T. BP_{ND} was assessed using the Logan's reference tissue model (t* = 30 min; (Logan et al., 1996)) with the cerebellum as reference region and based on average k2', resulting from the simplified reference tissue model. V_T in the equilibrium (between 50 and 100 min) equals the radioligand concentration in tissue target region (C_T; kBq*cm^{-3}) to plasma activity (C_p; kBq*mL^{-1}) ratio ($V_T = C_T/C_p$; (Elmenhorst et al., 2007b)). VOIs were grouped into the following anatomical regions: frontal lobe, striatum, pallidum, thalamus, insula, anterior cingulate cortex, posterior cingulate cortex, occipital lobe, hippocampus, amygdala, temporal lobe, parietal lobe, cerebellum and cerebellar lobules Crus I/II (hemispheric extensions of lobule VIIA; (Stoodley and Schmahmann, 2010)), brainstem and vermis, as well as cerebellum (reference region). V_T values were determined for all 15 brain regions, since a reliable calculation is also possible for the reference region (cerebellum) and low binding regions (cerebellum and cerebellar lobules Crus I/II, brainstem and vermis) due to blood sampling and detection of parent tracer in plasma. BP_{ND} values cannot be assessed for the reference region and reliably determined in low binding regions.

2.5. Statistical Analyses

Demographic and scan characteristics were compared between females and males using independent samples *t*-test and Mann-Whitney U test for normally and non-normally distributed data, respectively. The significance level was set at $p < 0.05$ in all statistical tests. Distribution of rs5751876 allele variants between sexes were

compared using Chi-squared test. The effect of sex on BP_{ND} and V_T values was analysed using one-way multivariate analysis of variance (MANOVA) with regions as dependent variables and sex as fixed factor. Post hoc independent samples t-tests were performed within each region. P values for BP_{ND} and V_T were corrected for multiple comparisons using Bonferroni-Holm method. Statistical power was indicated by the effect size using Cohen's d (Hojat and Xu, 2004).

Self-reported habitual time to go to sleep, self-reported habitual sleep duration, self-reported habitual sleep latency, habitual midpoint of sleep, and PSG-recorded sleep period time (SPT), sleep latency, sleep efficiency ([total sleep time/time in bed]*100), rapid-eye-movement (REM) sleep, time spent in NREM sleep stage 1 (N1; transition between waking and sleeping), 2 (N2; stable sleep), and 3 (N3; deep sleep, SWS), and wakefulness (Richard B Berry et al., 2017; R. B. Berry et al., 2017), percentage of SPT (%SPT) occupied by N1, N2, N3, REM, and wakefulness, as well as time spent in N3 in the first NREM/REM sleep cycle were compared between females and males using independent samples *t*-test and Mann-Whitney U test for normally and non-normally distributed data, respectively. Self-reported sleep duration and PSG-recorded TST as well as self-reported and PSG-recorded sleep latency were compared using paired t-test in females and males. The relationship between sleep latency, sleep efficiency, total time spent in N3, time spent in N3 in the first NREM/REM sleep cycle and regional BP_{ND} and V_T values was examined for each sex by calculating Spearman's rho.

3. Results

3.1. Demographic and scan characteristics

Details regarding participants and PET scans are given in Table 1. Female and male participants did not differ in age or genotype regarding rs5751876 allele variants. Males had higher BMI compared to females. The injected dose of [^{18}F]CPFPX per kilogram was higher in female participants compared to male participants. Scan start times, specific activities at injection time, and masses of injected radioligand did not differ between sexes.

3.2. A_1AR availability in females and males

3.2.1. Females have higher BP_{ND} values than males

An effect of sex on regional BP_{ND} values was yielded by the one-way MANOVA ($F_{12, 37} = 3.293$, $p = 0.003$, Wilk's $\Lambda = 0.484$), which included twelve brain regions. Post hoc comparison revealed significantly higher BP_{ND} values in females compared to males in all brain regions ($ps_{corr.} < 0.025$, $ds > 0.73$; Table 2, Fig. 1a). The largest differences between BP_{ND} values of females and males were found in the pallidum and the anterior cingulate

cortex amounting to 29 %. The smallest difference was found in the occipital lobe, where the mean BP_{ND} value was higher by 12 % in females. Averaged parametric images of female and male participants are depicted in a planar and a surface representation in Fig. 2a and Fig. 2b, respectively.

3.2.2. V_T values show no sex-related difference

The one-way MANOVA, including 15 brain regions, showed a difference between females and males on V_T values ($F_{15, 34} = 3.178$, $p = 0.003$, Wilk's $\Lambda = 0.416$). Independent sample t-test post hoc analysis on regional V_T values revealed no significant differences between females and males in all regions ($ps_{corr.} > 0.999$, $ds < 0.09$; Table 3, Fig. 1b). Fig. 3a and Fig. 3b show representative averaged parametric images of both female and male participants in a planar and a surface representation, respectively.

3.3. Relationship between sleep and A_1AR availability

3.3.1. Sleep efficiency correlated positively and sleep latency negatively with BP_{ND} values in females

PSG sleep recordings of one female participant and one male participant could not be fully scored due to technical problems and were therefore not included in the analysis. N3 %SPT was higher in females compared to males (Table 4), whereas the time spent in sleep stage N1 was higher in males compared to females. All other sleep data recorded the night before the PET scans did not differ between the sexes. Self-reported habitual sleep latency was shorter and habitual midpoint of sleep was earlier in female participants compared to male participants. Self-reported habitual time to go to sleep and self-reported habitual sleep duration did not differ between females and males. Self-reported sleep duration was longer than PSG-recorded SPT in both females ($p = 0.001$) and males ($p = 0.013$). Self-reported sleep latency was shorter compared to PSG-recorded sleep latency in females ($p = 0.016$), but not in males ($p = 0.055$).

In female participants, time spent in N3 in the first NREM/REM sleep cycle, defined according to the rules of Feinberg and Floyd (Feinberg and Floyd, 1979), correlated positively with BP_{ND} values of the frontal lobe (Supplementary Fig. 1a), parietal lobe (Supplementary Fig. 1b), striatum, and posterior cingulate cortex (Spearman's rho ranging from -0.459 in the posterior cingulate cortex to -0.583 in the frontal lobe, $p \leq 0.048$; Supplementary Table 1). Positive correlations were found between sleep efficiency and BP_{ND} in almost all brain regions in females, excluding anterior cingulum and temporal lobe (Spearman's rho ranging from 0.458 in the hippocampus to 0.814 in the striatum, $p \leq 0.049$; Supplementary Fig. 1c-d). In addition, sleep latency correlated negatively with BP_{ND} in nearly all brain regions, except the hippocampus, occipital, and parietal lobe (Spearman's rho ranging from -0.451 in anterior cingulate cortex to -0.630 in the pallidum, $p \leq 0.046$). This was

strongly driven by a single subject who had a particularly long latency (133.5 min) and low BP_{ND} values. Without this subject, however, the correlations in the pallidum and posterior cingulate cortex were still significant (Spearman's rho = -0.569 and -0.498, p = 0.011 and 0.030, respectively; Supplementary Fig.1e-f). Time spent in N3 did not correlate with BP_{ND} values of females. None of these sleep variables correlated with BP_{ND} values of males.

3.3.2. Sleep efficiency correlated positively with V_T values in females

In female participants, sleep efficiency correlated positively with V_T values in all brain regions, except the region cerebellum and cerebellar lobules Crus I/II (Spearman's rho ranging from 0.470 in the cerebellum without vermis to 0.618 in the striatum, $p \leq 0.042$; Supplementary Fig. 2 and Supplementary Table 2). None of the other sleep variables correlated with V_T values in females and males, excluding time spent in N3 and V_T of the parietal lobe (Spearman's rho = 0.382, p = 0.041) in males.

4. Discussion

We examined sex differences in the A_1AR availability in the human brain under well-rested conditions. Our main finding were higher [^{18}F]CPFPX BP_{ND} values in the brains of females compared to males. These differences occurred in all investigated brain regions in a homogeneous pattern pointing to a sex-specific predisposition. This view is supported by a report about different A_1AR expression in another organ than the brain. Female mice showed 25 % higher cardiac A_1AR expression compared to males (McIntosh et al., 2010). Furthermore, our experimental settings aimed excluding functional effects on A_1AR availability by assuring well-rested conditions. Thus, a potential bias by varying sleep duration was excluded. Too little sleep was shown to increase A_1AR density and mRNA levels in rats (Basheer et al., 2001; Elmenhorst et al., 2009) and A_1AR availability in humans (Elmenhorst et al., 2017; Elmenhorst et al., 2007b). Autoradiography studies on post-mortem brain slices did not find sex differences in A_1AR binding in the temporal cortex (Glass et al., 1996) and hippocampus (Ułas et al., 1993) which might be related to small sample sizes (n = 8 (3 females) (Glass et al., 1996) and n = 10 (3 females) (Ułas et al., 1993)) and higher average age. Age is an important factor because A_1AR availability was shown to decrease with age (Meyer et al., 2007). This might also explain why the very same study did not find sex differences in a study population with a wide age range that included only 9 participants (4 females) in the same age range as our subjects (Meyer et al., 2007). In contrast, our data corroborates a previous PET study focussing on genetic variation in a group of comparable age in a subset of

anxiety-related brain areas (Hohoff et al., 2020). They found 25 % and 21 % higher A_1AR availability based on BP_{ND} in the amygdala and thalamus, respectively, in females compared to males. All other brain regions were on average 11 % higher in females than in males but did not reach significance (personal correspondence with the authors of (Hohoff et al., 2020).

4.1. Sex differences in brain receptor systems

We found sex differences in A_1AR availability in terms of the BP_{ND} in all regions. Sex differences have also been reported in other receptor systems and brain chemistry (Cosgrove et al., 2007). In female participants, 5-HTT availability in the diencephalon and brainstem (Staley et al., 2001), cerebral 5-HT_{1A} receptor availability (Jovanovic et al., 2008), and 5-HT levels in whole blood and plasma (Ortiz et al., 1988) were higher than in male participants. Higher dopamine transporter and dopamine D_2-like receptor binding potentials were observed in female participants (Kaasinen et al., 2001; Lavalaye et al., 2000; Staley et al., 2001). Midbrain dopamine D_2 receptor availability was higher in female smokers compared to male smokers (Okita et al., 2016). In females, higher numbers of cortical muscarinic acetylcholine receptors were found (Yoshida et al., 2000), but lower metabotropic glutamate type 5 (mGlu5) receptor binding potentials (Smart et al., 2019). All cited PET findings have been reported in terms of BP_{ND}, but not in terms of V_T.

4.2. Impact of gonadal hormones on brain receptor availability

It is possible that sex differences in A_1AR availability are related to gonadal hormones, especially oestrogen. In oestrogen-positive MCF-7 cells, a human breast cancer cell line, an upregulation of A_1ARs, A_{2A}ARs, and A_3ARs was induced by the oestrogen agonist 17β-estradiol (Mohamadi et al., 2018). It was assumed that oestrogen receptors mediated this effect (Mohamadi et al., 2018). In rats, differences in concentration and frequency of spontaneous adenosine release were shown among brain regions and between sexes (Borgus et al., 2020). In addition, an influence of the oestrous cycle phase on spontaneous adenosine release was found (Borgus et al., 2020). Borgus et al hypothesized that such findings might be due to sex differences in the adenosine receptor density (Borgus et al., 2020) because both A_1AR and A_{2A}AR control spontaneous adenosine release (Nguyen et al., 2014; Wang and Venton, 2017). However, the adenosine level should have no effect on A_1AR availability, as there is no evidence that endogenous adenosine displaces [^{18}F]CPFPX *in vivo* (Elmenhorst et al., 2007b). Furthermore, even regional receptor availability can change with the oestrous cycle. In female cynomolgus monkeys, 12 % higher D_2 receptor availability was found in putamen and caudate nucleus in the luteal phase compared to the follicular phase (Czoty et al., 2009). Autoradiography with a 5-HT_{1A} receptor agonist in female rats showed that the number of binding sites measured as B_{max} was increased by 18 fmol/mg in the ventromedial

hypothalamic nucleus during oestrus compared to dioestrus (Flügge et al., 1999). Interestingly, progesterone fluctuates during the menstrual cycle and a negative relationship between serum allopregnanolone levels, a progesterone metabolite, and serotonin transporter availability in the prefrontal cortex, pallidostriatum, insula, hippocampus, and posterior cingulate were found in female participants (Sundström Poromaa et al., 2018). However, receptor availability may not in fact fluctuate with the menstrual cycle. Imaging of the mGlu5 receptor revealed higher BP_{ND} values in male participants compared to female participants, but the receptor availability remained constant across menstrual phases (Smart et al., 2019). Changes in ovarian hormone levels can affect neurotransmitter systems and behaviour. In healthy females, estradiol concentration decreased to menopausal level following gonadotropin-releasing hormone agonist implant injection. This decrease which was associated with a rise in depressive symptoms (Frokjaer et al., 2015). In addition, a positive correlation was found between increased depressive symptoms and increased neocortical serotonin transporter availability (Frokjaer et al., 2015). Nevertheless, the gradual and temporal influence of oestrogen on cerebral A_1AR availability should be subject to further investigations.

4.3. Relationship between sleep and A_1AR availability

The A_1AR plays a modulatory role in the sleep-wake cycle and sleep-wake homeostasis (for review see (Basheer et al., 2004; Huang et al., 2011)). It is, therefore, not surprising that sex has an influence on both sleep and the availability of cerebral A_1AR. The female sex is associated with greater total sleep time (Goel et al., 2005; Ohayon et al., 2004), greater percentage of REM sleep and SWS (Ohayon et al., 2004), greater slow-wave activity (SWA) amplitudes (Armitage et al., 2000), shorter sleep latency (Goel et al., 2005), earlier ideal sleep onset time (Tonetti et al., 2008), longer ideal sleep duration (Tonetti et al., 2008), and a better sleep efficiency compared to men (Goel et al., 2005). In agreement with this notion, we confirmed a greater percentage of SWS (N3 %SPT) in female participants of our sample when compared to male participants, as well as lower total duration of superficial stage N1 in females. In female participants, analyses showed positive correlations between N3 sleep in the first NREM/REM sleep cycle and frontal and striatal BP_{ND} values. SWA, predominantly occurring during N3 sleep, is highest at the beginning of the sleep period, i.e., in the first and second NREM/REM sleep cycle, and decreases progressively (Feinberg, 1974; Williams et al., 1964). Higher sleep efficiency as well as lower sleep latency were associated with higher regional BP_{ND} values in some brain regions. Sleep efficiency also correlated positively with V_T in all brain regions in females. In male participants, no correlations between sleep data and BP_{ND} and V_T values were found, except time spent in N3 and V_T in the parietal lobe. Longer SWS and shorter sleep latency indicate a higher homeostatic sleep pressure, as was found

after sleep deprivation ((Borbély et al., 1981) and reviewed by (Dijk and Landolt, 2019)). Since longer N3 sleep in the first NREM/REM sleep cycle and shorter sleep latency correlated with higher BP_{ND} in female participants but not in male participants, this could suggest that females live under higher homeostatic sleep pressure than males even under well-rested conditions. In conclusion, the results could indicate that sex differences in the sleep characteristics may be partially explained by differences in the A_1AR availability between females and males.

4.4. Limitations

The present study has some methodological limitations. The injected dose of [^{18}F]CPFPX per kilogram was higher in female participants than in male participants. However, the injected dose per kilogram was not correlated with BP_{ND} or V_T values in any region ($p > 0.609$). This indicates that the A_1AR availability did not depend on the injected dose of [^{18}F]CPFPX. Therefore, the injected dose of [^{18}F]CPFPX per kilogram did not affect our results and was not included in the analyses. The same applies to the BMI. Although males had a higher BMI compared to females, BMI did not correlate with BP_{ND}, V_T and sleep variables, except time spent in N3 and N3 %SPT, which were negatively correlated ($-r \leq 0.469$, $p \geq 0.001$). Consequently, the assumptions for a multivariate analysis of covariance were not met and the BMI was not included as covariate in the analyses (Kraemer, 2015). Notably, there was no difference in the mass of injected CPFPX, which is decisive for the occupancy of the receptors by the radioligand.

PET data were obtained from subjects participating in two different studies, but all scans were performed after 8 hours of scheduled sleep and at approximately the same circadian time in the afternoon. The different durations that participants of study #1 (8 nights) and study #2 (3 nights) spent in the sleep lab before the PET scan did not influence our results because all of them adapted their sleep behaviour one week before arriving in the sleep lab according to the sleep satiation protocol. No significant differences in sleep variables (except time spent in N1 and N1 %SPT) were observed in the second baseline night (night 3) between the two studies suggesting that the one-week sleep satiation protocol and the time to adapt to the sleep lab were sufficient to compensate for potential difference in previous sleep behaviour. Additionally, comparison (paired t-test) of sleep variables between the second baseline night (night 3) and the night before the scan (night 8) of study #1 did not reveal differences indicating that sleep behaviour did not change during the time in the sleep lab. Consequently, our data indicated that the duration spent in the sleep lab did not influence sleep behaviour. Particularly, since we found no differences in sleep latency and N3 sleep, this also indicates that there was no change in sleep pressure that could affect A_1AR availability. Furthermore, when excluding participants from study #1 from our analysis, we still found higher BP_{ND} values in females compared to males (e.g., amygdala: 0.52 ± 0.10 (f), 0.40 ± 0.11

(m); frontal lobe: 0.64 ± 0.08 (f), 0.53 ± 0.08 (m); $p < 0.05$). This indicates that the habituation to the lab environment did not affect our results regarding cerebral A_1AR availability. Therefore, pooling the two datasets from study #1 and #2 should not have impacted the results.

It is possible that the genotypes of the study participants might have influenced our findings. Three participants of study #1 were rs5751876 C/C homozygous, one was T/T homozygous, nine were C/T heterozygous, whereas two were not genotyped. These genotype frequencies nearly represent the normal distribution of rs5751876 allele variants in the population (Janik et al., 2015; Kobayashi et al., 2010; Rogers et al., 2010). By contrast, twenty-eight participants of study #2 were C/C homozygous, one was T/T homozygous, and six were C/T heterozygous. Genetic variants in both A_1AR and A_{2A}AR genes, *ADORA1* and *ADORA2A*, respectively, impact cerebral A_1AR availability and distribution (Hohoff et al., 2014). Rs5751876 T-allele carrier (C/T + T/T) had higher A_1AR availability in all brain regions compared to C/C homozygotes (Hohoff et al., 2014). However, the genotype distributions in our investigation groups did not differ significantly (Table 1). Thus, genetic differences should not have affected overall results. Nevertheless, the generalisability of the findings is limited by the pre-selection of the genotype in study #2.

Our results regarding sex differences in A_1AR availability were not influenced by potential sex differences in caffeine and alcohol consumption and withdrawal effects. Comparison (independent sample t-test) of habitual alcohol and caffeine consumption between female and male participants did not reveal any significant difference. Furthermore, only participants reporting no alcohol or drug abuse as well as a habitual caffeine consumption below 450 mg/day were included in the studies and all participants had to abstain from any consumption of caffeine and alcohol for one week before arriving in the sleep lab and during their time in the lab.

[^{18}F]CPFPX BP_{ND} revealed sex differences in A_1AR availability but these sex differences could not be confirmed on the basis of V_T. One possible reason for this finding could be that BP_{ND} only includes specific binding of the tracer, as it refers to the ratio of specifically bound radioligand to that of non-displaceable one (Innis et al., 2007), whereas V_T also includes unspecific binding, as it is the ratio of the concentration of radioligand in tissue, containing specifically and non-specifically bound as well as free radioligand, to that in plasma (Innis et al., 2007). The unspecific binding might mask sex differences in A_1AR availability. We did not observe differences of V_T values between females and males in the cerebellum, which was considered to be a suitable reference region with low specific binding of [^{18}F]CPFPX (Bauer et al., 2003). Therefore, it can be concluded that the concentration of non-displaceable radioligand did not differ between females and males, hence this did not account for the different results of V_T and BP_{ND} analyses. Possible sex-specific differences in

411 the specific binding of [^{18}F]CPFPX in the reference region are alleviated in the resulting BP_{ND} (Elmenhorst et al.,
412 2007a). Test-retest analysis of [^{18}F]CPFPX results demonstrated their reproducibility and reliability, but showed
413 that non-invasive outcome parameters were superior to invasive ones (Elmenhorst et al., 2007a). Although we
414 found no significant difference in A_1AR availability in the reference region (cerebellum) between male and
415 female participants based on V_T, the percentage difference between the cerebellar mean values suggests that
416 A_1AR availability might be slightly higher in males than in females. This could bias our BP_{ND} results in the
417 opposite direction. However, this is highly speculative due to the current insufficient data availability.

418 Furthermore, it is unlikely that the results are affected by the performed template-based attenuation correction
419 (Izquierdo-Garcia et al., 2014). Neither Izquierdo-Garcia (2014) nor Ladefoged (2017) reported concerns about
420 the potential sexual dimorphism on the heads. The performed attenuation correction should handle sex-
421 dependent variability in head geometry and skull thickness but likely fails to accurately reflect changes in bone
422 density. In contrast to the Boston method by Izquierdo-Garcia (2014), the Resolute method from Copenhagen
423 (Ladefoged et al., 2015) considers bone density. However, the results of those two methods did not differ
424 significantly (Ladefoged et al., 2017). Moreover, the cohort of the atlas-based method (Izquierdo-Garcia et al.,
425 2014) is young enough (mean age ± SD 53.5 ± 12.7; personal correspondence with D. Izquierdo-Garcia) that it
426 could well correspond to the bone density of our cohort. No differences were found in bone thickness at the level
427 of the cerebellum (point D) between white females and males aged 20 to 40 (Adeloye et al., 1975). No
428 systematic difference in the slope of the plasma input functions was found between female and male participants,
429 making systematic differences in liver metabolism unlikely.

430 As already discussed, the oestrogen level in females might contribute to sex differences in the A_1AR availability.
431 In the present study, oestrogen levels were not measured and only in a subset of subjects, the last menstrual
432 period was recorded in the preliminary medical interview. In addition, some female participants were taking
433 contraceptives. Thus, the influence of oestrogen on A_1AR availability could not be considered.

434 Furthermore, participants had to follow a one-week sleep satiation protocol before arriving in the sleep lab to
435 exclude any confounding of the results by pre-existing sleep deficits, which are ubiquitous in our society, and
436 which are partially compensated for by longer sleep on free days (Roenneberg et al., 2003). The subject's
437 preferences were considered to a limited extent as they were allowed to choose a bedtime of 11 p.m. or 12 a.m.
438 Although conclusions drawn from objective and subjective measurements have to be treated cautiously, PSG-
439 recorded SPT was shorter compared to self-reported sleep duration suggesting that the sleep protocol is sufficient
440 for the subjects to participate in the studies well-rested without sleep deficit. This is supported by a shorter self-

reported habitual sleep latency compared to PSG-recorded sleep latency in females. However, the given fixed sleep protocol limited the significance of some sleep variables such as SPT and correlations based on them with A_1AR availability as sleep preferences (e.g., bedtime, sleep duration) were largely removed. It should be noted that sleep variables, A_1AR availability, and their relationships were investigated under well-rested conditions and may differ from real-life conditions. Females reported a shorter sleep latency compared to male subjects at home. This difference was not observed anymore during the lab phase (based on PSG recordings). Even though we included participants due to their preferred sleep timing, we cannot exclude that the imposed sleep schedule could have influenced our results.

4.5. Conclusion

In conclusion, our analysis gives a first indication for potential sex differences in A_1AR availability even under well-rested conditions. A_1AR availability as quantified by [^{18}F]CPFPX BP_{ND} is higher in females compared to males, whereas no such effect was evident on the basis of V_T. Considering the involvement of adenosine in sleep-wake control, our findings may partially explain some sleep characteristics in females. Our investigation may serve as a basis for further PET and autoradiography studies that specifically investigate cerebral sex differences in A_1AR availability in humans.

5. Declarations

5.1. Data and code availability statement

All MR and PET data analysed during the current studies are available from the corresponding author on reasonable request.

5.2. Declaration of Competing Interest

The authors declare that they have no conflict of interest.

5.3. Funding

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5.4. Ethics approval

All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The studies were approved by the Ethics Committee of the regional Medical Board (Ärztekammer Nordrein) and the German Federal Office for Radiation Protection.

5.5. Consent to participate

Informed consent was obtained from all individual participants included in the studies.

5.6. German Clinical Trial Registry

DRKS #DRKS00010194, registered 22 March 2016,
https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00010194

DRKS #DRKS00014379, registered 04 April 2018,
https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00014379

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8. Figure Captions

2-column fitting image

Fig. 1. A_1AR availability (a) was higher in females compared to males in terms of BP_{ND}, (b) but did not differ in terms of V_T. Differences between sexes were compared using MANOVA and post hoc independent sample *t*-test (*, $P < 0.05$; **, $P < 0.01$).

2-column fitting image

Fig. 2 Average images of [^{18}F]CPFPX BP_{ND} show higher A_1AR availability in females than in males. (a) Planar parametric images. Coordinates according to the Montreal Neurological Institute Brain Atlas were -2, -18, 14 (*x*, *y*, *z*). (b) Surface representations.

2-column fitting image

Fig. 3 Average images of [^{18}F]CPFPX V_T did not differ between females and males. (a) Planar parametric images. Coordinates according to the Montreal Neurological Institute Brain Atlas were -2, -18, 14 (*x*, *y*, *z*). (b) Surface representations.

9. Tables

Table 1. Participants and PET data

	Females (n = 20)	Males (n = 30)	*p*-value
Study affiliation	#1: n = 5 #2: n = 15	#1: n = 10 #2: n = 20	
Age [years]	26.9 ± 4.7	28.8 ± 5.6	0.249
BMI	21.8 ± 2.5	24.3 ± 2.2	0.0004
Weight [kg]	61.2 ± 9.9	81.6 ± 10.7	1.37E-08
Distribution of rs5751876 allele variants	70.0 % C.C 30.0 % C.T/T.T	60.7 % C.C 39.3 % C.T/T.T	$X^2(1) = 0.440$ p = 0.507 $\phi = 0.096$
Injected dose of [^{18}F]CPFPX per body weight [MBq/kg]	2.7 ± 0.5	2.3 ± 0.3	0.003
Specific activity at injection time [GBq/µmol]	103.6 ± 80.7	101.3 ± 67.2	0.984
Mass of injected CPFPX [nmol]	2.4 ± 1.5	2.8 ± 2.1	0.722
Injection time and scan start [hh:mm:ss]	14:38:14 ± 01:42:00	14:17:09 ± 01:47:59	0.452

Abbreviation: BMI, body mass index.

Values are given at mean ± SD; *p* values from independent samples *t*-tests (BMI, weight), Mann-Whitney U test (age, injected dose of [^{18}F]CPFPX per body weight, specific activity at injection time, mass of injected CPFPX, injection time and scan start) in the case of non-normality, or Chi-squared test (distribution of rs5751876 allele variants)

751 **Table 2.** Regional [^{18}F]CPFPX BP_{ND} values in females and males

Region	Females (n = 20)	Males (n = 30)	% Difference (females > males)	*p*-value	Holm's adjusted *p*-value	Cohen's d
Frontal lobe	0.63 ± 0.09	0.54 ± 0.12	17 %	0.005	0.018*	0.86
Striatum	0.69 ± 0.12	0.56 ± 0.13	24 %	0.001	0.004**	1.07
Pallidum	0.60 ± 0.10	0.47 ± 0.13	29 %	0.0003	0.003**	1.14
Thalamus	0.80 ± 0.11	0.66 ± 0.14	20 %	0.001	0.006**	1.02
Insula	0.65 ± 0.09	0.53 ± 0.12	23 %	0.001	0.004**	1.08
Anterior cingulate cortex	0.57 ± 0.11	0.45 ± 0.11	29 %	0.0002	0.003**	1.15
Posterior cingulate cortex	0.73 ± 0.09	0.63 ± 0.13	16 %	0.005	0.015*	0.85
Occipital lobe	0.79 ± 0.09	0.70 ± 0.13	12 %	0.012	0.025*	0.75
Hippocampus	0.55 ± 0.08	0.43 ± 0.11	27 %	0.0002	0.002**	1.16
Amygdala	0.51 ± 0.10	0.41 ± 0.11	25 %	0.002	0.010*	0.94
Temporal lobe	0.75 ± 0.07	0.65 ± 0.11	15 %	0.0004	0.004**	0.99
Parietal lobe	0.70 ± 0.11	0.61 ± 0.13	15 %	0.015	0.015*	0.73

752 *p*-values from post-hoc independent samples *t*-tests and corrected according to Bonferroni-Holm method (*, $P < 0.05$; **, $P < 0.01$).

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Table 3. Regional V_T values in females and males

Region	Females (n = 20)	Males (n = 30)	% Difference (females > males)	*p*-value	Holm's adjusted *p*-value	Cohen's d
Frontal lobe	0.98 ± 0.21	1.01 ± 0.19	-2.6 %	0.652	1.00	-0.13
Striatum	1.02 ± 0.23	1.01 ± 0.20	0.8 %	0.887	1.00	0.04
Pallidum	0.96 ± 0.20	0.94 ± 0.17	1.7 %	0.759	1.00	0.09
Thalamus	1.09 ± 0.22	1.09 ± 0.21	-0.1 %	0.986	1.00	-0.01
Insula	0.99 ± 0.20	0.99 ± 0.18	-0.002 %	1.000	1.00	-0.0001
Anterior cingulate cortex	0.94 ± 0.19	0.93 ± 0.17	1.1 %	0.852	1.00	0.05
Posterior cingulate cortex	1.04 ± 0.21	1.06 ± 0.19	-2.0 %	0.720	1.00	-0.10
Occipital lobe	1.08 ± 0.21	1.11 ± 0.18	-3.1 %	0.548	1.00	-0.17
Hippocampus	0.92 ± 0.18	0.92 ± 0.16	-0.2 %	0.974	1.00	-0.01
Amygdala	0.89 ± 0.19	0.89 ± 0.17	0.2 %	0.971	1.00	0.01
Temporal lobe	1.06 ± 0.20	1.08 ± 0.20	-2.4 %	0.660	1.00	-0.13
Parietal lobe	1.02 ± 0.21	1.05 ± 0.19	-2.6 %	0.630	1.00	-0.14
Cerebellum and cerebellar lobules Crus I/II	0.53 ± 0.10	0.59 ± 0.11	-9.2 %	0.086	1.00	-0.51
Brainstem and vermis	0.57 ± 0.12	0.59 ± 0.11	-4.1 %	0.452	1.00	-0.22
Cerebellum without vermis (ref. region)	0.55 ± 0.10	0.60 ± 0.11	-8.8 %	0.104	1.00	-0.48

p-values from post-hoc independent samples *t*-tests and corrected according to Bonferroni-Holm method (*, P < 0.05; **, P < 0.01).

Table 4. Sleep data

	Females (n = 19)	Males (n = 29)	*p*-value
Self-reported habitual time to go to sleep [hh:mm:ss]	23:16:19 ± 00:35:01	23:36:32 ± 00:43:10	0.121
Self-reported habitual sleep duration [min]	455.5 ± 52.0	460.3 ± 61.4	0.784
Self-reported habitual sleep latency [min]	11.1 ± 5.6	18.4 ± 10.3	0.012*
Habitual midpoint of sleep [hh:mm:ss]	03:15:09 ± 00:46:29	03:45:02 ± 00:46:26	0.038*
SPT [min]	445.1 ± 32.6	455.1 ± 15.4	0.470
Sleep latency [min]	27.1 ± 27.0	22.8 ± 12.2	0.929
Sleep efficiency [%]	87.1 ± 7.9	88.1 ± 4.8	0.605
Stage N1 [min]	14.8 ± 6.9	19.2 ± 6.8	0.038*
Stage N1, %SPT	3.4 ± 1.7	4.2 ± 1.5	0.079
Stage N2 [min]	197.3 ± 42.1	214.6 ± 28.8	0.096
Stage N2, %SPT	44.1 ± 8.0	47.1 ± 5.6	0.140
Stage N3 [min]	100.7 ± 25.3	85.3 ± 28.2	0.059
Stage N3, %SPT	22.8 ± 6.2	18.8 ± 6.3	0.034*
Stage N3 in 1st NREM/REM sleep cycle [min]	45.0 ± 17.6	39.0 ± 16.1	0.387
REM sleep [min]	105.0 ± 24.8	103.5 ± 21.8	0.941
REM, %SPT	23.6 ± 5.2	22.8 ± 4.8	0.776
Wakefulness [min]	27.0 ± 18.9	32.5 ± 18.3	0.067
Wakefulness, %SPT	6.1 ± 4.5	7.1 ± 4.1	0.086

Abbreviations: SPT, sleep period time; REM, rapid-eye-movement; NREM, non-rapid-eye-movement; N1, NREM sleep stage 1; N2, NREM sleep stage 2; N3, NREM sleep stage 3; %SPT, percentage of SPT.

Values are given at mean ± SD; *p*-values from independent samples *t*-tests (self-reported habitual time to go to sleep, self-reported habitual sleep duration, habitual midpoint of sleep, time spent in and percentage of SPT occupied by stage N1, N2, N3) or Mann-Whitney U test (SPT, sleep efficiency, time spent in N3 in first NREM/REM sleep cycle, time spent in REM sleep and wakefulness, percentage of SPT occupied by REM sleep and wakefulness) (*, $P < 0.05$).