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# Sleep-related and diurnal effects on brain diffusivity and cerebrospinal fluid flow

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ABSTRACT

The question of how waste products are cleared from the brain, and the role which sleep plays in this process, is critical for our understanding of a range of physical and mental illnesses. In rodents, both circadian and sleep-related processes appear to facilitate clearance of waste products. The purpose of this study was to investigate whether overnight changes in diffusivity, brain volumes, and cerebrospinal fluid flow measured with MRI are associated with sleep parameters from overnight high-density sleep EEG, and circadian markers. In healthy adults investigated with MRI before and after sleep EEG, we observed an increase in water diffusivity overnight, which was positively related to the proportion of total sleep time spent in rapid eye movement (REM) sleep, and negatively associated with the fraction of sleep time spent in non rapid eye movement (NREM) sleep. Diffusivity was also associated with the sleep midpoint, a circadian marker. CSF flow increased overnight; this increase was unrelated to sleep or diffusivity measures but was associated with circadian markers. These results provide evidence for both sleep related and diurnal effects on water compartmentalisation within the brain.

## 1. Introduction

Poor or insufficient sleep has been described as a hidden epidemic (Naiman, 2017), with far-reaching effects on physical and mental health. According to the Centers for Disease Control (CDC), only 35% of adults in the USA report sleeping a healthy seven hours per night, (2) and sleep deprivation has been associated with an increased risk of a myriad of diseases including cancer (Haus and Smolensky, 2013), obesity (Fatima et al., 2016), heart disease (Tobaldini et al., 2017), stroke (Palma et al., 2013), and various neuropsychiatric disorders (Kahn-Greene et al., 2007, Kalmbach et al., 2017). However, despite the critical importance of sleep for life and health, the functions underlying the restorative nature of sleep remain unclear.

In recent years it has been suggested that one of the functions of sleep may be to cleanse the brain of waste products which accumulate during waking hours. One hypothesis, the cerebral "glial lymphatic" or glymphatic hypothesis proposed in 2012 by Iliff et al, posits that the paravascular transport of cerebrospinal fluid (CSF) plays a critical role in cleaning the brain (Iliff et al., 2012). Other suggested pathways for waste clearance include carrier-mediated blood brain barrier (BBB) transport (Spector et al., 2015). While the relative importance of these pathways remains an open question, both glymphatic clearance and BBB transport have been reported to be modulated by sleep and circadian effects (Cuddapah et al., 2019), underscoring the importance of sleep and diurnal rhythms for clearing the brain of waste products.

Consistent with the glymphatic hypothesis, increases in CSF flow have been reported during sleep both in rodents (Xie et al., 2013) and in humans (Fultz et al., 2019). In rodents, the volume of the interstitial space was reported to increase by 60% during slow wave sleep (Xie et al., 2013). Circadian effects on CSF drainage or flow have also been observed, both in rodents, (Hablitz et al., 2020) and in humans (Nilsson et al., 1992), suggesting that clearance may be influenced by circadian as well as sleep-related effects. Sleep is regulated by the interaction of a sleep homeostatic process with a circadian process (Borbely and Achermann, 1999), which are closely linked and can be challenging to separate experimentally. However, a recent sleep deprivation study incorporating a control for circadian rhythmicity showed an accumulation of amyloid in healthy individuals after just one night of sleep deprivation (Shokri-Kojori et al., 2018), highlighting the importance of sleep for clearance of amyloid from the brain.

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Reports from both animal and human studies suggest that the phase of sleep characterized by high levels of slow wave activity (SWA) on electroencephalography (EEG), i.e. deep sleep, is likely to coincide with increased clearance of metabolic waste products from the brain (Xie et al., 2013), either via the glymphatic or other pathways. SWA can be quantified from the EEG power in the frequency range of 1–4.5 Hz measured during non-rapid eye movement (NREM) sleep, and is thought to be related to the restorative function of sleep, since it increases in a use-dependent manner with the time spent awake and decreases during the night (Borbely and Achermann, 1999). In humans, disruption of slow wave sleep leads to an increase in CSF amyloid levels (Ju et al., 2017), which appears to be specifically related to SWA, since other markers of sleep quality (e.g. sleep efficiency) and markers for sleep architecture (total sleep time, or the time spent in REM or NREM sleep) were not related to accumulation of amyloid. Therefore, SWA appears to be important for the clearance of waste products like amyloid from the brain, but SWA has not yet been directly linked to markers of glymphatic activity, either in animals or in humans.

Previous imaging studies have shown diurnal changes in T1-weighted MR signal or diffusivity throughout the day, potentially arising from changes in the CSF fraction within the imaging voxels, or shifts in water between the intracellular and extracellular compartments (Bernardi et al., 2016; Demiral et al., 2018; Elvsashagen et al., 2015; Nakamura et al., 2015; Thomas et al., 2018). However, since these studies did not include sleep EEG, it is not yet known how these changes in diffusivity or T1-weighted signal relate to the SWA, or markers for sleep architecture like the time spent in different stages of sleep. Another recent study showed changes in pulsatile motion of CSF during sleep in humans, assessed from changes in the EEG spectrogram, specifically a decrease in occipital alpha power and an increase in delta and theta power, during sleep (Fultz et al., 2019). However, this study did not examine the link between differences in CSF clearance between participants and corresponding between-subject differences in sleep structure (e.g. from the duration or fraction of time spent in different sleep stages), or SWA and CSF flow. The purpose of this study was to investigate whether overnight changes in diffusivity, brain volumes, and cerebrospinal fluid flow measured with MRI are associated with SWA (as a marker for sleep quality) and the time spent in REM and NREM sleep, reflecting sleep architecture, measured with overnight high-density sleep EEG. Based on the results from rodent studies reporting an increase in interstitial volume during slow wave sleep, we hypothesised that overnight changes in diffusivity would be present and would be related to the duration and intensity of slow wave sleep. Based on the glymphatic hypothesis, we further expected that overnight changes in CSF flow within the cerebral aqueduct would also be related to diffusivity changes and the duration and intensity of slow wave sleep. From previous publications reporting diurnal effects on CSF flow, we hypothesised that CSF flow and diffusivity would be related to circadian markers. Given the lack of previous studies examining the link between glymphatic markers and sleep architecture, we also performed an exploratory analysis to investigate whether glymphatic markers (diffusivity and CSF flow) would be related to sleep architecture, quantified from the fraction of total sleep time spent in REM and NREM sleep.

## 2. Materials and methods

### 2.1. Participant recruitment

The participant group consisted of 18 healthy young adult volunteers (8 female, mean age 21 years, range 18–24), recruited from local advertisements placed on University webpages. All participants were non-smokers with no family history of psychopathology, sleep disorders, or chronic illness, and with no current use of psychoactive agents or other medications. All were good sleepers (with a sleep efficiency > 80%), with a caffeine and alcohol intake limited to less than 160 mg/day and < 14 mg/day respectively. Participants had to refrain from alcohol use from 48 hours prior to the experiment, and from travelling across time zones for one month before the study. On the day of the imaging experiments, participants were also asked to refrain from extensive exercise or visits to a sauna. The study was approved by the cantonal ethics committee of Zürich and participants were compensated monetarily for their time.

### 2.2. Experimental protocol

Starting from one week prior to the MRI and EEG recordings, participants were instructed to keep a regular sleep-wake schedule according to their habitual bedtime, which was verified by sleep logs and wrist actigraphy (GENEActiv, activinsights Ltd., Kimbolton, Huntingdon, UK). On the night of the sleep EEG assessment, participants underwent an MR imaging investigation from approximately 2 h prior to their habitual midweek bedtime (starting 8:28 pm ± 8 min), after which the hd-EEG net was fitted and EEG was recorded all night. The following morning, the MRI protocol was repeated after removal of the EEG net, at 8:12 am (± 8 min). Blood pressure and pulse rate were recorded before and after each MRI session, and body mass (as a marker for hydration) was measured before and after the sleep EEG assessment.

Diffusion tensor imaging (DTI) data were collected using a pulsed gradient spin echo sequence with 35 unique sampling directions with a diffusion weighting of $b$ = 1000 s/mm$^2$, echo time (TE) = 77 ms, repetition time (TR) = 6000 ms, field of view = 27.8 cm, acquisition matrix = 96 × 96, reconstruction matrix = 256 × 256. Four b=0 images were also acquired, resulting in a scan time of four minutes. The MR imaging protocol also included a 3D high resolution T1 weighted IR-SPGR scan (TE = 3 ms, TR = 8 ms, inversion time = 600 ms, flip angle = 8°, voxel resolution = 1mm$^3$) for the assessment of brain and CSF volumes, a CINE peripheral pulse-gated, 2D phase contrast sequence for assessing CSF flow within the cerebral aqueduct (TE/TR = 5.1/8.6 ms, flip angle = 20°, Nex = 2, VENC = 10 cm/s, voxel resolution 0.8 × 0.8 × 4 mm$^{3)}$, and single voxel MR spectroscopy in the parietal lobe (Volk et al., 2018, Volk et al., 2019). All MR imaging data were acquired with a GE 3T MR750 MRI scanner (GE Healthcare, Waukesha, WI, USA).

### 2.3. EEG acquisition and analysis

EEG data were acquired with a high density (128 channel) EEG net (SensorNet, Electrocal Geodesic Inc., Eugene, Oregon, USA). Additional data were collected from two EMG electrodes and two electrodes at the earlobes (Grass Technologies, West Warwick, RI, USA). The nets were adjusted to the vertex and mastoids and electrodes were filled with electrolyte gel. All impedances were kept below 50 kΩ. Data were sampled at 500 Hz, referenced to the vertex electrode (Cz), and subsequently band-pass filtered (0.5–40 Hz) and downsampled to 128 Hz. Artefacts were removed by visual inspection if the mean power exceeded a fixed threshold (Volk et al., 2018). Channels with bad quality data and channels below the ears (frequently contaminated by muscle artefacts) were removed and data were re-referenced to the mean of the data from all good quality channels. Sleep stages were scored by two sleep experts according to standard criteria of the American Academy of Sleep Medicine (Iber et al., 2007). The duration of rapid eye movement (REM) and NREM sleep was calculated for each participant as a percentage of the total sleep duration. SWA was calculated for each electrode in all artefact-free N2 and N3 epochs as the mean spectral power in the range of 1–4.5 Hz (Fast Fourier Transformation, Hanning window, averages of five 4-second epochs). SWA of rejected channels was interpolated using spherical interpolation (Delorme and Makeig, 2004), resulting in SWA data from 109 channels for each subject. SWA was then averaged over every non-REM sleep episode within each sleep cycle, defined according to the criteria of Feinberg and Floyd (Feinberg and Floyd, 1979). The overnight change in SWA was calculated as the fractional decrease of SWA between the NREM sleep episode displaying maximal SWA (at the

beginning of the night) and the NREM sleep episode displaying minimum SWA (at the end of the night). For assessment of circadian effects, the sleep midpoint was calculated from the time of sleep onset plus one half of the sleep duration. In order to account for potential variability in sleep onset and offset times between the laboratory and home settings, sleep midpoint data were calculated from both the actimetry data and sleep logs (acquired at home before the EEG measurement) and across the EEG measurement night. The chronotype was calculated according to the Munich Chronotype Questionnaire (Frey et al., 2009; Roenneberg et al., 2004).

### 2.4. MRI data analysis

The 3D IR-SPGR T1-weighted images were segmented using the longitudinal pipeline (Reuter et al., 2012) in Freesurfer, (Fischl et al., 2002, 2004) and the total grey and white matter volumes were extracted from the relevant output files for the evening and morning scans. The total cerebrospinal fluid (CSF) volume was calculated for each scan as the sum of the volumes of the left and right lateral ventricles, the 3rd and 4th ventricles, the left and right choroid plexi, and the sulcal CSF from the Freesurfer segmentations. Differences in the total grey matter (including both cortical and subcortical grey matter), total white matter, and total CSF volumes between the evening and morning scans were tested with a paired t-test.

DTI images were skull-stripped and corrected for eddy current effects, and the diffusion tensor was fitted using the tools within the FSL software library (Smith et al., 2004). The fractional anisotropy maps were then normalized into MNI space using the registration tools incorporated into the tbss pipeline (Smith et al., 2006), and the same transformations were applied to the mean diffusivity (MD) maps to align them into standard space. The registration into standard space was performed both with a two-step method, registering the evening and morning scans for each participant together before normalising them into standard (MNI) space, and with a one-step registration, where each FA map was normalised independently to the MNI template. (For the two-step registration, the morning images for each participant were first linearly registered to the evening scans, using an affine registration with 12 degrees of freedom, before registering both sets of scans to a standard-space template using a combination of linear and nonlinear registrations, implemented within the default tbss pipeline. For the one-step method, both the morning and evening images were independently registered to the template using the standard (linear and nonlinear) registrations in tbss).

Differences in MD between the evening and morning scans were tested using the permutation testing methods implemented in FSL randomize, using a single group paired difference design, corrected for multiple comparisons using threshold free cluster enhancement (TFCE). Permutation testing was performed for all voxels within the brain mask, derived after skull-stripping the template image with the brain extraction tool (FSL BET). Data were then extracted from significant clusters for comparison with sleep parameters, including the fraction of time spent in REM and NREM sleep, the mean SWA over the night, and the overnight (percentage) change in SWA. Correlations between the overnight change in diffusivity and sleep parameters were performed using Pearson's correlation for normally distributed variables and Spearman's rho for non-normally distributed variables. *P*-values of $p < 0.05$ were considered significant.

In a second analysis, further voxelwise comparisons were conducted to identify brain regions where the evening and morning diffusivity correlated with the mean SWA or the overnight change in SWA, the sleep midpoint or chronotype. All voxelwise analyses were performed with FSL randomise, controlling for multiple comparisons with TFCE.

CSF flow data were analysed from 2D phase contrast images in the cerebral aqueduct, aligned perpendicular to the aqueduct axis. In-house software was used to manually define the aqueduct lumen boundary over time using both magnitude and phase images for placement. Forward and reverse flow rates were calculated by integration of velocity measures over the lumen area. Integration over time was used to define forward, reverse and net flow volumes over a cardiac cycle for each subject during both evening and morning scans.

Statistical analyses, apart from the voxelwise TBSS analyses, were performed with SPSS version 22. Differences in brain volumes, CSF flow, and baseline physiological measures (pulse, blood pressure, body weight) were performed with a paired t-test for normally distributed variables and a Wilcoxon signed-ranks test for non-normally distributed variables (Table 1).

## 3. Results

### 3.1. Overnight changes in diffusivity and brain volumes

At a corrected threshold of $p < 0.01$, both increases and decreases in mean diffusivity (MD) were observed between the evening and morning DTI scans. While the overnight decrease in MD was confined to clusters within the CSF spaces, the overnight increase was observed in a widespread network of regions across the brain, including bilateral clusters within the cerebral white matter, cingulate gyrus, basal ganglia, thalamus, cerebellum, and brainstem (Fig. 1). Both the overnight decrease in MD within the CSF spaces and the overnight increase in MD within the brain parenchyma appeared to be a stable effect across the participant group, with all 18 participants demonstrating comparable changes in the same direction within the significant clusters. However, these significant differences were only observed for the data registered with the two-step registration, but not when the evening and morning data were registered into standard space independently.

Segmentation of the 3D T1-weighted SPGR images from the evening and morning scans revealed a significant overnight increase in total grey matter volume ($p = 0.009$) and a trend towards a significant overnight increase in white matter volume ($p = 0.075$, paired *t*-test, see Table 1 for details). These results are consistent with the decrease in total brain volume from morning to evening reported previously (Nakamura et al., 2015; Thomas et al., 2018), although opposite effects have been reported after intense practice (Bernardi et al., 2016). Segmentation of the 3D T1-weighted images also revealed a significant overnight decrease in total CSF volume ($p < 0.001$), which was significantly associated with the overnight reduction in MD within the CSF spaces (Pearson's $R = 0.73$, $p = 0.001$), such that participants showing a larger overnight decrease in MD within CSF showed a larger overnight decrease in CSF volume.

### 3.2. Link between diffusivity and sleep and circadian parameters

Within the clusters demonstrating an overnight increase in MD within the brain parenchyma (displayed in Fig. 1), the percentage increase in MD correlated positively with the fraction of total sleep time spent in REM sleep and negatively with the fraction of time spent in NREM sleep (REM: Spearman's rho = 0.571, $p = 0.013$, NREM: Spearman's rho = -0.571, $p = 0.013$, see Table 2 for a summary of the sleep structure parameters for the participant group). The percentage increase in MD within the brain parenchyma was also negatively associated with the sleep midpoint from actimetry (Pearson's $R = -0.555$, $p = 0.032$), but not with the sleep midpoint from the EEG ($R = -0.309$, $p = 0.212$). No significant correlations were observed between the overnight increase in MD in the brain and SWA (mean SWA: Spearman's rho = -0.069, $p = 0.785$; overnight (%) change in SWA: rho = -0.187, $p = 0.458$), or the chronotype (rho = -0.004, $p = 0.987$).

Within the clusters showing an overnight decrease in MD within the CSF spaces, no significant associations were observed between the overnight reduction in MD and the fraction of time spent in REM or NREM sleep (REM: Pearson's $R = 0.23$, NREM: Pearson's $R = -0.23$, $p = 0.356$), SWA (mean SWA: Spearman's rho = 0.185, $p = 0.463$; overnight change in SWA: rho = 0.257, $p = 0.303$), the sleep midpoint

**Table 1**
Brain volumes, cerebrospinal fluid flow, and baseline physiological parameters measured in the evening and the following morning.

| | | evening | morning | |
|---|---|---|---|---|
| Brain volumes | Grey matter (mL) | 708 (58.3) | 713 (60.7) | 0.009* |
| | White matter (mL) | 456 (51.7) | 458 (53.2) | 0.075 |
| | CSF (mL) | 20.2 (7.1) | 19.6 (6.9) | <0.001* |
| Cerebrospinal fluid flow | Forward flow (ml/min) | 0.0551 (0.039) | 0.0707 (0.052) | 0.009*§ |
| | Reverse flow (ml/min) | 0.0336 (0.029) | 0.0433 (0.035) | 0.123§ |
| | Net flow (ml/min) | 0.0215 (0.019) | 0.0278 (0.032) | 0.268§ |
| Baseline physiological parameters | Pulse rate (bpm) | 67.2 (8.4) | 63.3 (8.8) | 0.070 |
| | Systolic blood pressure (mm Hg) | 118 (6.7) | 115 (11) | 0.116 |
| | Diastolic blood pressure (mm Hg) | 70.0 (11) | 72.7 (6.6) | 0.237 |
| | Body weight (kg) | 66.8 (9.1) | 66.7 (9.1) | 0.404 |

§ Wilcoxon signed ranks test

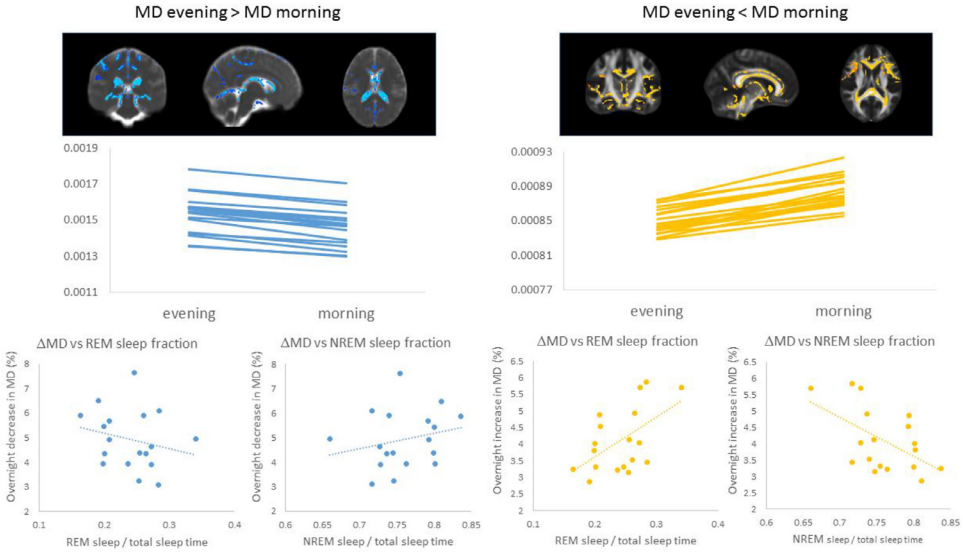


**Fig. 1.** Between the evening and morning scans the mean diffusivity (MD) decreased significantly within the CSF spaces (top left panel, $p < 0.01$, TFCE corrected) and increased significantly within a widespread network of brain regions (top right panel, $p < 0.01$, TFCE corrected), including the periventricular white matter, basal ganglia, thalamus, cingulate gyrus, cerebellum and brainstem. Within the clusters showing a significant overnight change in diffusivity, all 18 participants demonstrated a decrease in MD within the CSF (middle left panel) and an increase in MD in the brain (middle right panel). While the overnight decrease in MD within the CSF spaces was not significantly associated with the fraction of sleep time spent in REM or NREM sleep ($R$ = +/- 0.23, $p = 0.356$), the overnight increase in MD in brain was significantly positively associated with the fraction of time spent in REM sleep (Spearman's rho = 0.571, $p = 0.013$) and negatively associated with the proportion of time spent in NREM sleep (Spearman's rho = -0.571, $p = 0.013$).

**Table 2**
Sleep structure parameters.

| Parameter | Mean (SD) |
|---|---|
| Total time in bed (min) | 461 (24) |
| Total sleep time (min) | 427 (28) |
| Sleep latency (min) | 15.9 (9.9) |
| Sleep efficiency (%) | 92.5 (4.3) |
| Wake after sleep onset (min) | 22.6 (13) |
| NREM sleep (min) | 326 (21) |
| N1 (min) | 24.8 (11) |
| N2 (min) | 215 (37) |
| N3 (min) | 85.8 (33) |
| REM sleep (min) | 101 (23) |

(EEG: Spearman's rho = -0.152, $p = 0.548$, actimetry: rho = -0.268, $p = 0.334$), or the chronotype (rho = -0.020, $p = 0.938$).

Intercorrelations between the sleep midpoint data derived with EEG, actimetry, and the questionnaires showed a strong correlation between the sleep midpoint calculated from the sleep logs and actimetry ($R = 0.78$) and a moderate correlation between the questionnaire logs and EEG ($R = 0.51$), but only a weak correlation between the sleep midpoint from actimetry and that from the EEG ($R = 0.20$).

In the voxelwise correlation analysis, the sleep midpoint derived from actimetry (using the median of the midweek sleep midpoint values, available only for 15 participants) was positively correlated with the MD in the evening in a set of regions including the lateral ventricles, cerebellum, pallidum, thalamus, as well as the cerebral white matter (Fig. 2, $p < 0.05$, TFCE corrected). The sleep midpoint from the sleep logs (in whom data were available for all 18 participants) was positively associated with the evening MD within a small cluster in the right lateral ventricle, overlapping with the regions showing a significant association between MD and the sleep midpoint from actimetry (overlaid in yellow, Fig. 2, top panels).

In contrast, the sleep midpoint from the EEG showed a significant negative correlation with the MD in the morning across a network of brain regions mostly within the white matter (supplementary Fig. 1, $p < 0.05$, TFCE corrected), and a trend was observed in similar regions between the sleep midpoint from EEG and the MD in the evening ($p < 0.07$, TFCE corrected, supplementary Fig. 1). The morning MD in the cerebellum also showed a trend-level correlation with the overnight percentage change in SWA (p=0.05, TFCE corrected), but not the mean SWA. The association between the MD and chronotype was not significant ($p > 0.23$, TFCE corrected). The overnight change in MD (calculated by subtracting the morning MD maps for each participant from the evening MD maps, after normalisation into standard space) was also correlated with the sleep midpoint from actimetry, and this association was present for the MD maps normalised with both the one-step and two-step registrations (Fig. 3).

### *3.3. Overnight changes in CSF flow, and associations between CSF flow and circadian parameters*

Due to pulse triggering (n=3) problems, CSF flow data were only available from a subset of $n = 15$ participants. In this subgroup, the

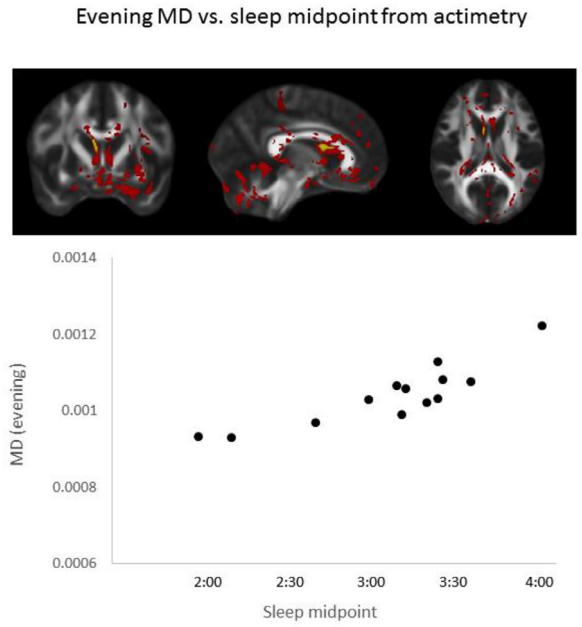


**Fig. 2.** The voxelwise correlation analysis between the baseline diffusivity and the sleep midpoint revealed a significant positive association between the weekday sleep midpoint from actimetry and the evening MD in a set of regions including the lateral ventricles, cerebellum, and diffuse clusters within cerebral white matter ($p < 0.05$, TFCE corrected). The sleep midpoint from the sleep logs showed a similar positive correlation with the MD within a small cluster in the right lateral ventricle (overlaid in yellow, $p < 0.05$, TFCE corrected).

forward flow (from superior to inferior) within the aqueduct was observed to increase significantly ($p = 0.009$, Wilcoxon signed ranks test) between the evening and morning scans, but there was no significant change in reverse or net flow within the aqueduct overnight ($p = 0.123$, $p = 0.268$, respectively, see Table 1 for details). The overnight change in forward flow was not significantly associated with the overnight change in diffusivity in the brain or CSF spaces, the SWA or the percentage of time spent in REM or NREM sleep (all $p > 0.45$). However, both the net CSF flow in the morning and the (nonsignificant) overnight change in reverse flow were related to the chronotype (rho = 0.53, $p = 0.04$).

## 4. Discussion

The question of how metabolic waste products are cleared from the brain, and the role which sleep and circadian effects play in this process, is a critical one with far-reaching importance for a range of physical and mental illnesses.

In the present study, we observed that the mean diffusivity of water in the brain and CSF spaces is significantly altered after a night of normal sleep in healthy adults, and is related to the sleep midpoint (a circadian marker). The observed change in diffusivity from evening to morning is consistent with the change in volume of the interstitial space reported during sleep in animals, and provides some evidence for changes in water compartmentalisation during sleep in humans. Importantly, we also show for the first time that this increase in diffusivity within the brain parenchyma between the evening and morning scans appears to be related to sleep. However, contrary to our hypotheses, the change in diffusivity was positively associated with the percentage of time spent in REM sleep, and not the intensity of slow wave sleep.

The positive correlation between the overnight change in MD and the relative time spent in REM (rather than NREM) sleep is surprising in the context of reports from animal studies suggesting that glymphatic activity increases during slow-wave NREM sleep (Xie et al., 2013). However, previous rodent studies did not distinguish between REM and NREM sleep states specifically, and since the relative duration of REM and NREM sleep are perfectly correlated, and interact closely with other parameters like the SWA, the effects of sleep on diffusivity may involve a complex interplay between various sleep parameters. The link between overnight changes in diffusivity within the brain and the proportion of total sleep time spent in REM sleep would fit with the apparent association between REM sleep loss and an increased risk of physical and mental illness (Naiman, 2017), and may be related to the increased pulse and respiration rate during REM sleep (Cajochen et al., 1994; Somers et al., 1993), but future studies involving simultaneous MRI and EEG during REM and NREM sleep will be needed to confirm the potential role of REM sleep in glymphatic clearance.

While the diffusivity within the brain parenchyma appeared to *increase* overnight, within the CSF spaces, a corresponding *decrease* in diffusivity overnight was observed. This overnight reduction in MD within the CSF may be related to an overnight decrease in temperature, since the diffusivity in ventricular CSF has been suggested to represent a marker for brain temperature (Hasan et al., 2015), and since the body temperature has been reported to decrease overnight, (Mackowiak et al., 1992) reaching a nadir between 4 and 6 am (Krauchi, 2002). However, this decrease in diffusivity within CSF was also related to a significant overnight decrease in CSF volume, as described previously (Thomas et al., 2018; Trefler et al., 2016). The circulatory dynamics of CSF are complex (for a recent review see: (Johanson et al., 2008)), but the factors which may account for changes in CSF volume include physiological changes like heartbeat, respiration, and posture, as well

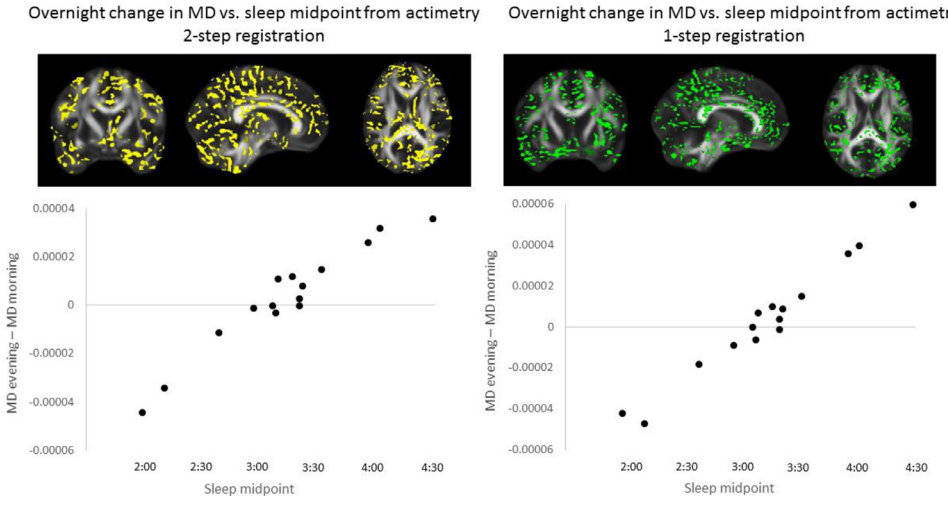


**Fig. 3.** The overnight change in MD was significantly correlated with the sleep midpoint from actimetry ($p < 0.05$, TFCE corrected), such that participants showing a larger overnight decrease in diffusivity (i.e. a larger diffusivity in the evening and/or a smaller diffusivity in the morning) tended to have a later sleep midpoint. This pattern was observed for MD maps normalised into standard space with both the two-step and one-step registrations (left and right panels, respectively).

as diurnal factors like the time of day (Spector et al., 2015). In addition to these hydrodynamic factors, hemodynamic factors, such as choroid plexus flow and pulsatility, neuroendocrine factors, transcription factors, transport enzymes, ion transporters, etc. are all thought to play a role in CSF dynamics (formation, flow turnover, reabsorption, etc.) (Johanson et al., 2008) Given the multiplicity of inter-related factors affecting CSF dynamics, it is challenging to identify the factors underlying this apparent change in volume, which could be due either to an increased rate of CSF reabsorption/turnover or reduced formation. However, since the participants in whom the diffusivity in CSF decreases the most also show the largest overnight changes in CSF volume, if the change in CSF diffusivity arises from a change in temperature, possibly due to circadian effects, then both the CSF volume and diffusivity might be affected by factors with a common circadian origin.

In parallel with the overnight decrease in CSF diffusivity and volume, we also observed an overnight increase in forward flow within the cerebral aqueduct. The overnight change in forward flow was not related to sleep or diurnal markers, although the (nonsignificant) overnight change in net flow and the morning net flow were related to circadian markers, consistent with previous reports of circadian effects on CSF flow (Nilsson et al., 1992; Kervezee et al., 2014). Taken together, the diffusivity and flow results provide some evidence that both sleep and circadian effects influence the distribution of water between the intracellular and extracellular compartments, and circadian effects also seem to influence CSF flow. However, the contribution of circadian factors to the observed changes in CSF dynamics and diffusivity should be further explored in future studies including additional objective markers for circadian effects, like dim light melatonin levels.

Contrary to our hypothesis, there was no apparent link between the overnight change in diffusivity and either the mean or overnight change in SWA. However, the diffusivity within a small, midline cluster in the cerebellum in the morning showed a trend towards an association with the overnight change in SWA ($p = 0.05$, TFCE corrected). At a weaker statistical threshold the extent of this cluster within the cerebellum expanded to include lateral regions, still localised within the cerebellum at $p = 0.08$. The observation of an apparent association between overnight changes in SWA and the diffusivity in the cerebellum in the morning is interesting in the light of reports from rodent studies that glymphatic clearance is enhanced in the cerebellum (Iliff et al., 2013), and is also consistent with results from a previous human study investigating changes in diffusivity with sleep deprivation (Demiral et al., 2018). However, the diffusivity also appears to be affected by diurnal effects, although the direction and significance of this finding varied with the method of assessing the sleep midpoint (from EEG, actimetry, or sleep diaries), as well as between brain regions. The lack of a perfect correlation between the various measurements of the sleep midpoint underscores the difficulties inherent in these measurements, since the sleep midpoint would ideally be measured on days when participants are on a regular sleep schedule and wake up spontaneously each morning. In the present study, the EEG measurement was timed to correspond with the midweek schedule because the weekend sleep schedule appeared to be more variable. However, $N = 14$ of the 18 participants regularly woke up to an alarm during the week, so the sleep midpoint from all three measurements (sleep logs, actimetry, and the EEG measurement which was timed to coincide with the participants' midweek schedules) could be an underestimate of the true circadian sleep midpoint. In addition, the data from the sleep logs is more subjective, and does not account for variations in sleep latency between participants. The sleep midpoint from actimetry should correspond to the sleep midpoint from the onset of N1 sleep, but is very dependent on the algorithm used to assess the actimetry data. For the assessment of circadian effects on diffusivity and flow in future studies, additional markers for circadian rhythmicity like dim light melatonin levels may provide important objective markers for the circadian timepoint for each measurement.

In addition to circadian effects, possible confounds relating to dehydration and other baseline physiological parameters should also be considered. We attempted to control for dehydration effects by weighing the participants before and after sleep recordings, and there was no apparent relationship between the overnight change in diffusivity or brain volumes and the change in weight (all $p > 0.3$). However, since the timing of fluid intake overnight and in the morning was not controlled, the weight measurements may not fully reflect hydration levels. The collection of urine samples before each MRI examination may be helpful for assessing hydration levels in future studies. Other baseline physiological parameters like pulse and blood pressure did not appear to be related to the observed changes in diffusivity (data not shown). However, since both the baseline and overnight change in diffusivity appear to be influenced both by sleep-related and circadian effects, these effects will need to be carefully separated in future studies, in order to understand the effects of sleep on glymphatic clearance in humans.

From a methodological standpoint, it is surprising that the overnight changes in diffusivity were only statistically significant when the diffusivity maps were registered into MNI space with a two-step registration, by first registering the maps for the evening and morning scans for each subject, before normalising these maps into standard space. One possible explanation for this observation is that the diffusivity changes reflect brain volume changes, which are corrected with the one-step registration to standard space. However, the affine registration between the evening and morning scans should also account for linear changes in brain volume within each participant between the two time points. Separating changes in water compartmentalisation from changes in volume experimentally with MRI is challenging since the water compartmentalisation affects the MR signal used to determine the fractional tissue volume within each voxel. However, the observation that the overnight changes in diffusivity within the brain parenchyma were unrelated to the changes in grey matter, white matter, or CSF volume suggests that the diffusivity changes may not be exclusively a reflection of brain volume changes. The significant correlation between the sleep midpoint from actimetry and the overnight change in diffusivity, calculated with the MD maps normalised into standard space with both the 1-step and 2-step registrations, provides some evidence that the diurnal change in diffusivity appears to be independent of the method used for normalisation of the images into standard space. An alternative explanation for the difference in the evening vs. morning results derived with the one-step and two-step registrations is that the one-step registration adds variance to the sample, since the nonlinear registration is applied twice to the data from each participant (from the morning and evening, respectively), potentially adding variability to the data and reducing the statistical power. However, future studies with a larger dataset would be needed to clarify whether the same effects are statistically significant in a larger sample, in which repeated samples from each participant were each normalised directly to standard space without an intermediate registration step.

While the present study provides the first indication for a potential role of REM sleep in water compartmentalisation within the brain, one important limitation to bear in mind when interpreting this data is that since the sleep EEG and MRI measurements were non-simultaneous, based on the present results it is not possible to confirm the existence of diffusivity changes *during* sleep. It may be the case that the diffusivity (and the volume of the interstitium) fluctuates during different sleep stages and returns to near baseline-levels upon waking. In this case, the observed link between overnight changes in MD and the fraction of sleep time spent in REM sleep would just reflect a residual change, which may or may not reflect the magnitude of the changes taking place during sleep. In addition, since the diffusion imaging protocol included just a single b-value, the diffusivity was quantified with a single compartment model, and the fast and slow diffusion could not be quantified separately. A dual compartment analysis would be interesting to include in future studies, although previous studies have reported that a mono-exponential tensor model is also sensitive to diffusivity changes within regions thought to be important for glymphatic clearance (Thomas et al., 2018). Another important limitation is the small sample size, and the as-

sociated risk of false positive or false negative results. This limitation is particularly important for the CSF flow analyses and the analyses of the actimetry data, where data was only available from a subset of 15 participants. The results should therefore be considered with caution until they can be replicated in a larger sample.

## Data for reference

The ethical approval granted to the authors by the IRB does not allow the publication of the raw data online. If readers would like to re-analyze the data set, additional ethical approval will be required. The authors would be happy to support additional ethical approval applications from researchers for access to this data set.

## Credit authorship contribution statement

**Ruth O'Gorman Tuura:** Conceptualization, Formal analysis, Methodology, Resources, Software, Supervision, Visualization, Writing – original draft. **Carina Volk:** Conceptualization, Project administration, Data curation, Formal analysis, Writing – review & editing. **Fraser Callaghan:** Methodology, Formal analysis, Software, Writing – review & editing. **Valeria Jaramillo:** Data curation, Writing – review & editing. **Reto Huber:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2021.118420.

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