Sampling issues of cerebrospinal fluid and plasma monoamines: Investigation of the circadian rhythm and rostrocaudal concentration gradient

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ABSTRACT

Biomarkers for neurodegenerative dementias offer interesting prospects regarding diagnosis and disease monitoring. Monoamines such as dopamine, (nor)adrenaline, serotonin (5-hydroxytryptamine or 5-HT), and their respective metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylglycol (MHPG), and 5-hydroxyindoleacetic acid (5-HIAA), were shown to be altered in dementia, including Alzheimer's disease (AD). Biomarker research is hampered by potential confounds including the influence of time of day and volume of cerebrospinal fluid (CSF) collected. Therefore, the possibility of a circadian rhythm in CSF and plasma, and the presence of a rostrocaudal concentration gradient (RCG) in CSF for aforementioned monoamines/metabolites, were investigated.

Circadian rhythmicity was assessed using reversed-phase ultra-high performance liquid chromatography with electrochemical detection (RP-UHPLC-ECD) to measure monoamine/metabolite concentrations in 271 paired CSF and plasma samples, successively collected over a period of 30 h and derived from eight healthy subjects. Plasma samples were also analyzed for melatonin, serving as positive control analyte, using ELISA. The RCG examination entailed RP-UHPLC-ECD analyses on five consecutive CSF samples derived from 10 patients with AD and 10 non-AD/control subjects.

Besides a diurnal rhythm for melatonin, we found a similar rhythmicity for plasma HVA, with acrophases occurring between 02:00 and 06:00 h, in four out of seven subjects. Three and two subjects showed a circadian rhythm for CSF HVA and 5-HIAA, respectively. No rhythmicity was observed in any other compound. We found that only CSF MHPG, HVA and 5-HIAA levels differed across CSF fractions, and that these changes in 5-HIAA levels varied in the AD versus non-AD/control group. Positive correlations between CSF volume and HVA and 5-HIAA levels, indicative of a RCG, were also observed. Such a RCG could not be detected for the other monoamines/metabolites. Our results stress the importance of standardizing sampling procedures of biological fluids with respect to time of day, volume and number of samples.

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Abbreviations

(N)A	(nor)adrenaline
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
AD	Alzheimer's disease
Aβ	amyloid beta
C1-5	cerebrospinal fluid fraction 1–5
CONTR	control
CSF	cerebrospinal fluid
DA	dopamine
DLB	dementia with Lewy bodies
DOPAC	3,4-dihydroxyphenylacetic acid
ELISA	enzyme-linked immunosorbent assay
HVA	homovanillic acid
LP	lumbar puncture
MESOR	midline estimating statistic of rhythm
MHPG	3-methoxy-4-hydroxyphenylglycol
P-tau$_{181P}$	tau phosphorylated at threonine 181
RCG	rostrocaudal concentration gradient
RP-UHPLC-ECD	reversed-phase ultra-high performance liquid chromatography with electrochemical detection
SCN	suprachiasmatic nucleus
TH	tyrosine hydroxylase
T-tau	total tau

1. Introduction

1.1. Monoamines as biomarkers for neurodegenerative dementias

Cerebrospinal fluid (CSF)- and blood-based biomarkers represent interesting opportunities for the differential diagnosis of neurodegenerative dementias (Lewczuk et al., 2018). For example, amyloid beta peptide of 42 amino acids (Aβ$_{1-42}$), total tau (T-tau) and tau phosphorylated at threonine 181 (P-Tau$_{181P}$) are widely known as the standard CSF biomarker panel to aid in the differential diagnosis of Alzheimer's disease (AD) (Dubois et al., 2014; Simonsen et al., 2017). Besides their application in diagnostic evaluation, biomarkers are implemented in other contexts of use, such as disease- and treatment response monitoring (Zverova, 2018). In this aspect, blood-based biomarkers are preferred over CSF markers given the relative invasiveness and practical disadvantages inherent to a lumbar puncture (LP) (Humpel, 2011; Zverova, 2018). Nevertheless, a suitable blood biomarker for AD diagnosis or disease monitoring, is still lacking. Some promising candidate molecules (plasma T-tau and serum neurofilament light chain) have already been identified (reviewed by Keshavan et al., 2017), but still face several challenges, among which are a lack of reproducibility (O'Bryant et al., 2017; Shi et al., 2018).

In addition, despite their usefulness for the diagnosis of AD, CSF Aβ$_{1-42}$, T-tau and P-Tau$_{181P}$ were proven to have little efficacy in diagnosing dementias other than AD (De Deyn, 2015; Engelborghs et al., 2008; Niemantsverdriet et al., 2015, 2017). However, distinct dementia types were previously hypothesized to have dissimilar neurochemical underpinnings with regard to monoamine content in the central nervous system, including dopamine (DA), (nor)adrenaline ((N)A), and serotonin or 5-hydroxytryptamine (5-HT), and their respective metabolites, i.e. 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and, 5-hydroxyindoleacetic acid (5-HIAA) (Vermeiren et al., 2016). Studies investigating brain areas of patients suffering from AD, frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB) support this theory (Vermeiren et al., 2015, 2016). These dissimilarities in monoaminergic compounds between AD and other dementias, have also been identified in CSF and serum (Aerts et al., 2011; Herbert et al., 2014; Janssens et al., 2018; Parnetti et al., 1992; Sjögren et al., 1998). For instance, the addition of CSF (and serum) MHPG - the major metabolite of (N)A indicating noradrenergic turnover (Chase et al., 1973) - to the standard CSF biomarker panel for AD, improved the ability of the panel to differentiate AD and DLB (Herbert et al., 2014; Janssens et al., 2018). Taken together, monoamines and/or their metabolites in CSF and blood are potentially of added value as diagnostic biomarkers for dementia, whether they are combined with a previously validated biomarker panel, or implemented as stand-alone compounds (Janssens et al., 2018).

1.2. Difficulties associated with sampling of CSF and plasma biomarkers

One of the difficulties concerning biomarker research, is the lack of reproducibility across laboratories (Lewczuk et al., 2018). The pre-analytical phase, comprising sample collection, -handling, -transport, and -storage, is reported to be specifically prone to errors, accounting for up to 70% of all mistakes in laboratory diagnostics (Lippi et al., 2011; Plebani and Carraro, 1997), and possibly impacting the quality of biomarker assessment (Lewczuk et al., 2018). Efforts should be made to develop a standardized and holistic approach covering the use of correct laboratory equipment, sampling and storage volume, appropriate temperature for transport and storage, centrifugation settings and fasting status of study participants (Lewczuk et al., 2018; Lippi et al., 2011; O'Bryant et al., 2015; Shi et al., 2018).

Another possible source of variability between the findings of different groups is the potential influence of time of day and volume of CSF aspirated. This work will primarily focus on sampling time, with respect to the circadian rhythm of monoamines and/or their metabolites in CSF and plasma, and the presence of a possible rostrocaudal concentration gradient (RCG) of these compounds in CSF.

1.2.1. Circadian rhythm of monoaminergic compounds in CSF and plasma

While some studies suggest the existence of a physiological circadian rhythm of CSF Aβ levels (Bateman et al., 2007; Bjerke et al., 2010; Huang et al., 2012), little evidence exists concerning possible circadian fluctuations of monoaminergic compounds in human (patho)physiology. Dopaminergic compounds, including DA and HVA, were found to fluctuate diurnally in the CSF of patients suffering from Parkinson's disease, restless legs syndrome and control subjects (Poceta et al., 2009). Alternatively, a circadian rhythm for 5-HT was reported in serum derived from healthy and schizophrenic subjects (Rao et al., 1994), as well as in whole blood samples of controls and depressed patients (Pietraszek et al., 1991). The main metabolite of 5-HT, 5-HIAA, was also shown to have a circadian rhythm contrary to that of its precursor in whole blood (Pietraszek et al., 1991). Diurnal fluctuations of plasma and serum (N)A have also been reported (Linsell et al., 1985; Rao et al., 1995), although other studies found an ultradian rhythm in the noradrenergic neurotransmitter system, consisting of 20–30 pulses of (N)A per 24 h (hrs), depending on the analysis software (Schöfl et al., 1997). Finally, concentrations of plasma MHPG were also shown to vary diurnally in six control subjects (DeMet et al., 1985). These findings could also be corroborated by a later study investigating plasma MHPG levels in healthy volunteers as well as in patients suffering from depression (Gwirtsman et al., 1989).

1.2.2. Rostrocaudal concentration gradient

Whereas limited evidence exists concerning diurnal rhythms of monoamines/metabolites in biological fluids, state of the art evidence generally agrees on the existence of a RCG for CSF HVA and 5-HIAA. A RCG of these compounds was indeed observed in healthy adult control

subjects as well as in patients suffering from neuropsychiatric diseases, such as vascular dementia, mixed dementia, organic depression and psychosis, which did not affect CSF circulation. In contrast, MHPG levels did not show any variation in consecutive CSF samples (Blennow et al., 1993). Similar findings were reported in a study analyzing monoaminergic metabolites in CSF derived from children and adolescents aged 6.50–17.25 years with obsessive-compulsive disorder and/or attention deficit disorder (Kruesi et al., 1988). In addition, levels of HVA and 5-HIAA in consecutive CSF fractions derived from patients with (possible) hydrocephalus also rose with increasing sampling volume in two separate studies (Malm et al., 1994; Sjöström et al., 1975). Concentrations of MHPG were previously reported to be comparable across CSF fractions (Kruesi et al., 1988; Sjöström et al., 1975), although another study reported increased MHPG levels with increasing CSF sampling volume (Ziegler et al., 1977). However, current literature provides little support for a RCG in levels of monoamine neurotransmitters. Most of the aforementioned studies also date from two decades ago, and analytical research methods have improved ever since.

1.3. Aims and hypothesis

The aim of this work is to elaborate on the presence of a possible circadian rhythm in plasma and CSF monoamines and their metabolites to establish an optimal sampling time of these biological fluids. We included plasma melatonin as a positive control analyte, given the extensive evidence of its circadian rhythm (Cajochen et al., 2003; Zeitzer et al., 2007; Zhdanova et al., 1998; Zisapel, 2018). Based on the available literature, we expected to find a circadian rhythm in plasma and CSF for 5-HT and 5-HIAA in particular, since these compounds are closely related to melatonin. In addition, we intended to provide evidence for a RCG of monoamine neurotransmitters and their metabolites, with special regard to HVA and 5-HIAA.

2. Materials & methods

2.1. Study population

The inclusion procedure for the study of circadian rhythm has been described in detail in previous work (den Daas et al., 2013; Naude et al., 2017). In short, eight healthy males aged 50–75 were enrolled, of whom seven completed the entire protocol. The exclusion parameters for this study entailed a body mass index $> 27 \, \text{kg} \, \text{m}^{-2}$, body weight $> 100 \, \text{kg}$, acute cellulitis, onychomycosis of the feet, increased white blood cell count, poor venous access, history of lower back pain, significant kyphosis and/or scoliosis or other spinal column deformities, persisting fibromyalgia, a history of difficult LP, a history of and/or any sign or symptom indicating abnormal hemostasis or blood dyscrasia and any disorder which could lead to increased intracranial pressure, such as glaucoma or hydrocephalus-related pathology. Elderly men were chosen since they have lower intradural cavity pressure and thus have a reduced likelihood of CSF leakage and post dural puncture headache. After a screening period, the subjects arrived at the clinic on day -2. During the next day (day -1), the volunteers received 2 L of 0.9% saline solution by intravenous infusion. On day 1, intradural catheterization was performed under strict aseptic conditions by an

experienced anesthesiologist. At 12 h and 36 h after intradural catheterization, the subjects received fraxiparine for antithrombotic prophylaxis. During this period, subjects were allowed to move around freely and samples of 2 mL each were collected in 5 mL polypropylene tubes by interval sampling over a 30-h period starting at 10:00 h (t = 1) of day 1 (Fig. 1). Samples were withdrawn at hourly intervals by aspiration with a syringe during the first 12 h, at four-hour intervals between sampling times 13 and 16, and, finally, at two-hour intervals from sampling time 16 until 19 (Fig. 1). This led to a total of 19 paired CSF and plasma serial samples per subject, resulting in a total of 271 CSF and plasma samples. This study was approved by the local medical ethics committee (Stichting Beoordeling Ethiek Bio-Medisch Onderzoek, Assen, the Netherlands) and conducted in compliance with the Helsinki declaration and the Guideline for Good Clinical practice.

For investigation of the RCG, 10 probable AD patients and 10 probable non-AD/control subjects were included via a study protocol concerning CSF biomarkers of dementia (Vermeiren et al., 2013). The diagnosis of probable AD was routinely made according to the revised criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al., 2011). Additionally, all AD patients fulfilled the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV-TR) (American Psychiatric Association, 2000). The clinically diagnosed AD patients underwent a LP and neurocognitive assessment including the Mini-Mental State Examination (Folstein et al., 1983) as part of their diagnostic work-up of probable dementia. In addition, an AD-specific, pathological CSF biomarker profile was determined in these patients shortly thereafter (i.e. CSF levels of $A\beta_{1-42} < 638.5 \, \text{pg/mL}$, T-tau $> 296.5 \, \text{pg/mL}$, and P-tau$_{181P} > 56.5 \, \text{pg/mL}$ (Engelborghs et al., 2008). Similarly, age- and gender-matched individuals who had subjective memory complaints and/or were referred to a neurologist on the suspicion of dementia, underwent LP and neurocognitive assessment. Although these 10 subjects had normal, physiological biomarker profiles, a non-AD dementia diagnosis could not be fully excluded solely based upon the measured $A\beta_{1-42}$, T-tau and P-tau$_{181P}$ levels. All included subjects were recruited at the Memory Clinic of the Hospital Network Antwerp Middelheim (ZNA) and Hoge Beuken (Antwerp, Belgium). According to the aforementioned protocol, five consecutive fractions of CSF were collected from both the non-AD/control and AD groups (Vermeiren et al., 2013). Lumbar puncture was performed at the L3/L4 or L4/L5 interspace between 08:00 and 10:00 h, after fasting and abstention from smoking for at least 12 h. Approximately 16.5 mL of CSF was collected in an ordered manner: C1 (4.5 mL), C2 (1.5 mL), C3 (1.5 mL), C4 (4.5 mL) and C5 (4.5 mL) fractions were sampled in polypropylene vials (Nalgene; VWR, Leuven, Belgium), making up a total of 100 samples for the RCG analysis of CFS monoamines. Samples were placed in liquid nitrogen immediately after LP, and stored at $-80 \, °\text{C}$ until neurochemical analysis. For routine AD CSF biomarker analyses, the C2 fraction was used. Biomarkers were measured via single analyte ELISA-kits (INNOTEST, Fujirebio, Ghent, Belgium).

2.2. RP-UHPLC-ECD

The quantification of CSF and plasma monoamines and their corresponding metabolites was performed using an optimized Alexys
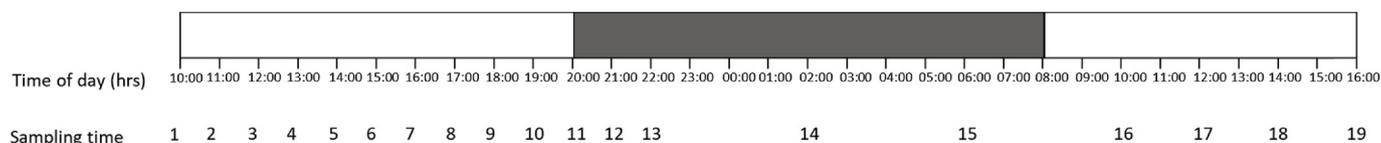

Fig. 1. Time schedule of CSF and plasma sampling. Sampling started at 10:00 h on day 1, and lasted until 16:00 h on day 2. During the first 12 h, CSF and plasma were collected hourly, followed by three four-hour sampling intervals. For the last six hours of the protocol, samples were taken every two hrs. Abbreviations: CSF: cerebrospinal fluid; hrs: hours.

Table 1
Demographics of the subjects included for the study of circadian rhythm. Data are represented as median, with the IQR denoted between brackets. Abbreviations: BMI: body mass index; IQR: interquartile range.

Parameter	
Age (years)	62.5 (8.3)
Gender (male/female)	8/0
Weight (kg)	79.4 (9.5)
Height (cm)	179.0 (16.3)
BMI (kg/m^2)	24.3 (2.6)

Neurotransmitter Analyzer with electrochemical detection (ECD) (Antec Leyden BV, Zoeterwoude, the Netherlands). This reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) system operated at an isocratic flow rate of 75 µL/min. The Decade II electrochemical detector was equipped with a thin layered electrochemical VT03 flow cell fitted with a glassy carbon 0.7 mm working electrode and an in situ Ag/AgCl (ISAAC) reference electrode. Integration of chromatograms was performed with channel integration M018/EN25B Clarity software (DataApex Ltd., Prague, The Czech Republic, version 6.2). The mobile phase consisted of 11% methanol combined with citric acid (100 mM), phosphoric acid (100 mM), octane-1-sulfonic acid sodium salt (2.8 mM), KCl (8 mM), and, ethylenediaminetetraacetic acid (0.1 mM). The pH of the mobile phase was set at 3.0. Samples of 5 µL

were loaded with an Alexys AS 110 Autosampler. Separation was achieved using a short 15 cm Waters Acquity Column (BEH C18, 1 mm diameter, particle size 1.7 µm), delivering optimal performances for monoamine analysis. Total runtime for each sample was under 15 min, after which all eight monoamines and metabolites were detected. With respect to the sample preparation protocol, Amicon Ultracentrifugal filters (3000 Da; Millipore, Ireland) were washed twice by centrifugation of 450 µL of sample preparation buffer at $14,000 \times g$ for 25 min at 4 °C. Next, 400 µL of plasma or CSF was directly transferred to the washed Amicon filters and centrifuged at $14,000 \times g$ for 40 min, still at 4 °C. After centrifugation, one fraction of the eluate was diluted 1:4 and injected in the RP-UHPLC-ECD system in the case of both CSF and plasma, while a second 1:20 plasma dilution was also analyzed.

2.3. ELISA

Enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions (RE54021; IBL. International GMBH, Hamburg, Germany) to determine plasma melatonin levels.

2.4. Statistics

For the analysis of circadian rhythm, mixed linear model analysis implementing compound symmetry covariance structure with heterogeneous variances, was performed on log-transformed plasma melatonin and plasma/CSF monoamine concentrations. Parameter
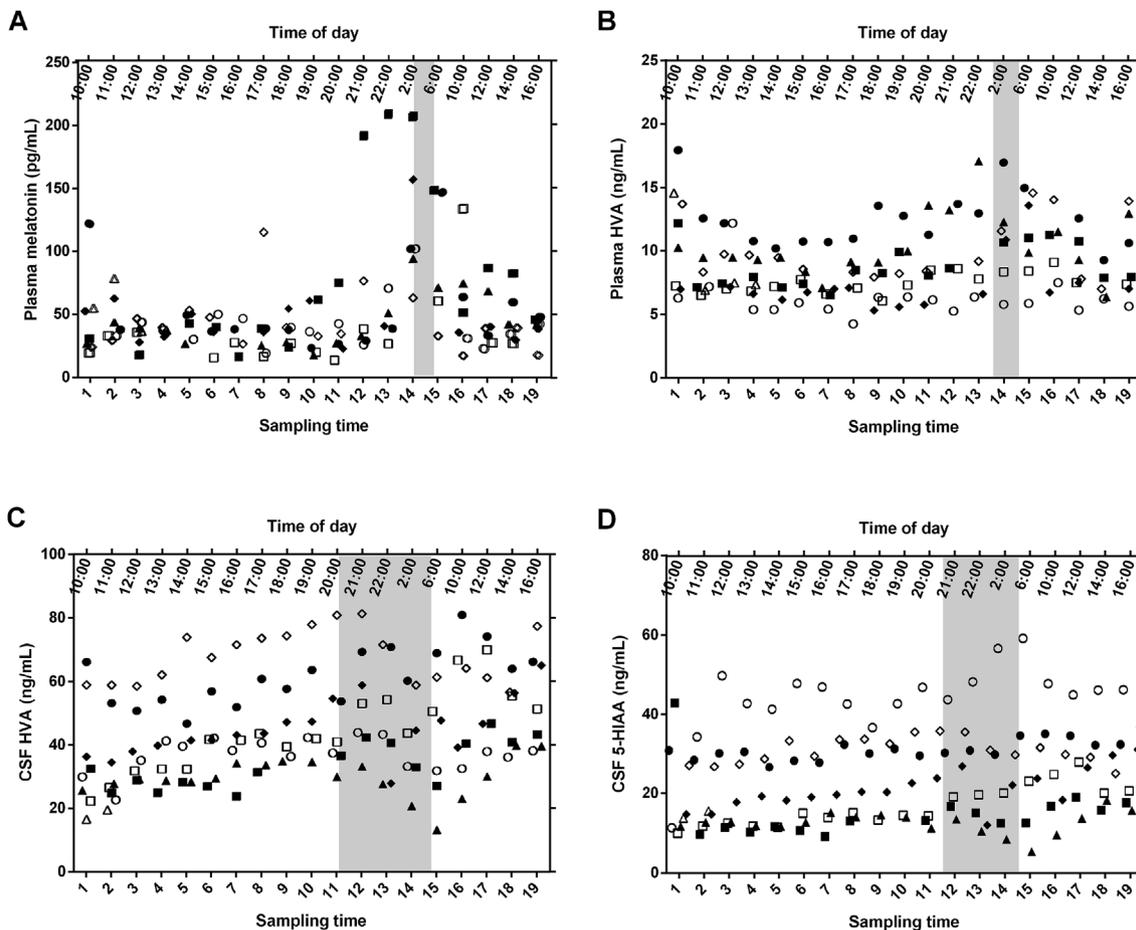

Fig. 2. Plasma melatonin and HVA concentrations, as well as CSF HVA and 5-HIAA levels, as a function of sampling time (panels A, B, C, and D respectively). The range of statistically significant acrophases for each compound, is indicated in grey. A: The plasma melatonin concentration at time point 14 differed significantly compared to almost all other time points. B: The acrophase of plasma HVA is situated in a similar time frame compared to melatonin. C, D: CSF HVA and 5-HIAA levels did not show a clear diurnal rhythm, and were characterized by a rather high variability. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; CSF: cerebrospinal fluid; HVA: homovanillic acid.

estimation for these models was obtained implementing the restricted maximum likelihood method. In case a statistically significant effect of time was detected, post-hoc analysis consisted of pairwise comparisons based on the least-squares means, with Bonferroni-adjusted p-values (statistically significant if $P < 0.05$). In addition, single cosinor analysis was applied to estimate the acrophase, amplitude and midline estimating statistic of rhythm (MESOR) of circadian rhythms displayed by melatonin and monoaminergic compounds. For the investigation of the RCG in CSF monoamines and/or metabolites, the independent-samples T-test and chi-square statistics were applied to verify if AD- and non-AD/CONTR groups were age- and gender-matched, respectively. In addition, mixed factorial ANOVA with CSF fraction as within-subjects variable and diagnostic class as between-subjects factor, was used to analyze the effects of the possible RCG and/or disease status on CSF monoamine/metabolite levels. In case the assumption of sphericity was violated, the Greenhouse-Geisser correction was applied. Post-hoc pairwise comparisons based on the estimated marginal means were performed to detect differences in monoamine/metabolite levels between CSF fractions. Again, p-values were adjusted according to the Bonferroni correction for multiple comparisons. Finally, the correlation between withdrawn CSF volume and concentrations of monoaminergic compounds was tested using Spearman's correlation statistics. All statistical analyses, except for cosinor analysis, were performed using SPSS 24.0 for Windows (IBM SPSS Software, Armonk, NY, IBM Corp.). Cosinor models were fitted using the cosinor package in R (Sachs, 2014), version 3.4.0 for Windows, while the zero-amplitude test for the detection of a statistically significant rhythm was performed using the cosinor2 package (Mutak, 2017). Figures were produced using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. Results

3.1. Demography – circadian rhythm

The demographic characteristics of subjects included for the study of circadian rhythm, are summarized in Table 1. Plasma and CSF samples were stored at −70 °C within 30 min after sampling, and were thawed twice beforehand to facilitate assays for other substances (den Daas et al., 2013).

3.2. Mixed linear model analysis to detect concentration changes over time

The concentration changes of plasma melatonin and CSF and plasma HVA over time, are depicted in Fig. 2. Mixed linear model analysis on log-transformed melatonin values indicated a statistically significant effect of sampling time (F (18, 24.79) = 3.61; $P < 0.05$), with pronounced post-hoc differences between time point 14 of sampling, corresponding to 02:00 h, and almost all other time points (Table 2). In addition, log-transformed HVA concentrations in CSF (F (18, 23.39) = 5.16; $P \leq 0.001$), as well as in plasma (F (18, 17.34) = 2.58; $P < 0.05$), varied significantly across time points. No differences could be detected in plasma HVA levels after correcting for multiple comparisons. As for log-transformed 5-HIAA levels in CSF, a significant effect of sampling time was also found (F (18, 21.61) = 2.15; $P < 0.05$), although only differences between the second and 17th, as well as between the second and 19th time points remained significant after Bonferroni correction (Table 2). Finally, a statistically significant difference across sampling times was detected for log-transformed plasma DOPAC levels (F (18, 14.30) = 2.48; $P < 0.05$), although only the pairwise comparison between the 17th and 18th time point remained significant after the post-hoc analysis. Statistically significant post-hoc differences in monoaminergic metabolite concentrations are summarized in Table 2, and raw data representing the mean concentrations at each significant time point, are represented in Supplementary Table 1.

3.3. Circadian rhythm analysis

A statistically significant diurnal rhythm could be corroborated for plasma melatonin in five out of seven subjects, while four out of seven subjects showed such a rhythmicity for plasma HVA. All estimated parameters and statistics for plasma melatonin and HVA in each subject are represented in Supplementary Table 2. The acrophase of plasma melatonin, indicating the time at which its concentrations were highest, was at night, ranging from about 02:00 to 05:30 h and corresponding to sampling point 14, for which most of the pairwise differences were found (Fig. 2, panel A; Table 2). A clear surge of plasma melatonin levels can indeed be observed at sampling time 14, followed by a rapid decrease of melatonin concentration. For two out of seven subjects, the assumption of normality of residuals was violated. In case of plasma HVA, four out of seven subjects showed a statistically significant diurnal rhythm, of which the acrophase ranged from about 01:00 to 04:30 h. Here, a more steady rise and decrease in analyte concentrations was observed (Fig. 2, panel B). However, the assumption of normality was not met for three individuals. No circadian rhythm could be observed in plasma DOPAC concentrations. Remarkably, a larger variability in CSF analyte levels was generally observed compared to plasma. Two out of seven subjects also showed a significant rhythm for CSF HVA, with peak levels at 04:53 h, while the acrophase of the third individual with a significant rhythmicity, was at around 20:00 h (Fig. 2, panel C). In addition, CSF HVA data of two subjects did not comply with the assumption of independence of residuals. Our results also indicated a 24-h rhythm in CSF 5-HIAA levels in two out of seven subjects, with acrophases ranging from 20:36–04:09 h (Fig. 2, panel D). Lastly, data of four subjects did not comply with the assumption of independent observations, and an additional non-normal distribution of CSF 5-HIAA levels was found in two of these four participants.

Table 2
Pairwise comparisons between sampling times of melatonin or monoaminergic compounds in CSF or plasma. Melatonin, HVA, 5-HIAA and DOPAC concentrations were log-transformed to fulfill the assumption of normality of residuals. Only statistically significant differences after post-hoc Bonferroni correction ($P < 0.05$) are displayed. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; CSF: cerebrospinal fluid; HVA: homovanillic acid; SE: standard error.

Parameter	Pairwise comparison (Sampling time)	Mean difference ± SE	p-value
Plasma Melatonin (Log(pg/mL))	2–14	−0.404 ± 0.089	0.036
	3–14	−0.508 ± 0.090	0.004
	4–14	−0.486 ± 0.072	0.001
	5–14	−0.438 ± 0.085	0.006
	6–14	−0.491 ± 0.096	0.005
	7–14	−0.569 ± 0.107	0.002
	8–14	−0.504 ± 0.121	0.031
	9–14	−0.500 ± 0.083	0.001
	10–14	−0.524 ± 0.101	0.003
	11–14	−0.548 ± 0.103	0.002
	14–17	0.423 ± 0.100	0.036
	14–18	0.415 ± 0.090	0.017
	14–19	0.479 ± 0.093	0.004
CSF HVA (Log(ng/mL))	2–10	−0.164 ± 0.036	0.044
	2–12	−0.205 ± 0.035	0.003
	2–17	−0.179 ± 0.037	0.006
	2–19	−0.208 ± 0.035	0.004
	3–12	−0.143 ± 0.029	0.005
	3–18	−0.116 ± 0.026	0.024
	3–19	−0.146 ± 0.028	0.002
CSF 5-HIAA (Log(ng/mL))	2–17	−0.172 ± 0.040	0.044
	2–19	−0.179 ± 0.040	0.030
Plasma DOPAC (Log (ng/mL))	17–18	0.129 ± 0.026	0.027

3.4. Demography – rostrocaudal concentration gradient

The study population consisted of age- and gender matched individuals in both non-AD/CONTR (n = 10) and AD (n = 10) groups. Demographic parameters are summarized in Table 3.

3.5. Mixed model ANOVA

Mixed model ANOVA was performed to verify effects of a possible RCG and diagnostic category on a subset of metabolite levels, as (N)A, DA, 5-HT and DOPAC could not be reliably detected in these CSF samples. A significant effect of CSF fraction was found for MHPG (F (2.66, 47.88) = 8.67; $P < 0.001$), while the effect of CSF fraction was not different between diagnostic classes (F (2.66, 47.88) = 1.52; $P > 0.05$). Likewise, HVA levels were found to differ across CSF fractions (F (2.32, 41.71) = 29.85; $P < 0.001$), without a significant interaction effect between diagnostic class and CSF subsample (F (2.32, 41.71) = 2.96; $P > 0.05$). A statistically significant effect of CSF fraction on 5-HIAA levels was also detected (F (2.56, 45.99) = 24.97; $P < 0.001$). Moreover, the behavior of the RCG was different in each diagnostic class, indicated by a significant interaction effect (F (2.56, 45.99) = 3.81; $P < 0.05$). The levels of monoamine metabolites in each subfraction and post-hoc differences, are displayed in Table 3. No global upward trend could be observed for CSF MHPG levels (Fig. 3, panel A), while diminished levels were noted in the C2 and C4 fraction. In addition, a similar pattern was detected for CSF HVA and 5-HIAA levels (Fig. 3, panel B and C, respectively), characterized by a general increase with collected CSF volume. A drop in both analyte levels could also be recognized in the C4 subsample. Lastly, markedly higher CSF 5-HIAA concentrations were observed in the AD group compared to non-AD/control individuals.

3.6. Correlation between metabolite concentrations and CSF fraction

Spearman's correlation statistics showed that both CSF HVA (rs (18) = 0.221; $P < 0.05$) and 5-HIAA (rs(18) = 0.213; $P < 0.05$) levels increased with collected CSF volume, whereas no association was found between MHPG levels and CSF fraction (rs(18) = 0.043; $P > 0.05$). As CSF fraction impacted CSF 5-HIAA levels differently in the non-AD/CONTR group compared to the AD group, we also investigated whether different correlations could be found in those two diagnostic categories. However, no statistically significant association was found between CSF 5-HIAA levels and CSF fraction when non-AD/CONTR (rs(8) = 0.211; $P > 0.05$) and AD (rs(8) = 0.254; $P > 0.05$) groups were analyzed separately.

4. Discussion

4.1. Circadian rhythm of melatonin and monoamines

Regarding circadian rhythm analyses in CSF, mainly negative results were found. Indeed, previous reports indicate the absence of diurnal rhythmicity in CSF DOPAC (Poceta et al., 2009) and MHPG, albeit in non-human primates (Perlow et al., 1978). Concentrations of CSF HVA and 5-HIAA did show a 24-h rhythm, although only in three and two subjects, respectively. Moreover, the acrophases of these rhythms showed a large variability, causing these results to remain inconclusive. Five and four out of the seven subjects who completed the protocol, showed a statistically significant circadian rhythm for plasma melatonin and HVA, respectively, which might be due to the non-normal distribution of these analyte concentrations. In addition, it is possible that the sleep-wake cycle of those two and three subjects was disturbed as a consequence of the hospital setting. Interestingly, the acrophases of melatonin and HVA, either in plasma or CSF, were situated in a similar time frame (01:00–06:00 h). This might be explained by the fact that both melatonin and DA are regulated by the major

circadian pacemaker in the hypothalamus, the suprachiasmatic nucleus (SCN) (Mendoza and Challet, 2014; Reiter, 1991; Sleipness et al., 2007; Zisapel, 2018). Briefly, the SCN regulates melatonin synthesis in the pineal gland via stimulatory and inhibitory stimuli, highly dependent on the light signal received from the retina (Kalsbeek et al., 2000; Perreau-Lenz et al., 2004). Dopaminergic activity is also hypothesized to be organized by the SCN. As an example, behavioral and neurochemical disturbances were observed in mice lacking Rev-erbα, a nuclear receptor involved in the feedforward loop of the molecular clockwork of circadian rhythm (Preitner et al., 2002). These mice exhibited increased production of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis (Molinoff and Axelrod, 1971), and increased DA turnover, indicated by a surge in HVA/DA ratios (Jager et al., 2014). In addition, protein levels of TH and DA transporters have been reported to be higher at night in the nucleus accumbens of male Sprague-Dawley rats (Sleipness et al., 2007), coinciding with our findings of peak HVA levels in both plasma and CSF at night. However, it should be noted that the evidence for dopaminergic regulation by the SCN was mainly raised in a preclinical setting, which might complicate a straightforward comparison with the results of this work. Our hypothesis stated that a circadian rhythm would be expected in the serotonergic system, as this is closely related to melatonin. However, no such diurnal variation was observed in these compounds, except for CSF 5-HIAA in only two out of seven participants. The relatively high variability of our measurements in combination with a small sample size, might account for these negative results.

We are also aware of the possible confounding influence of diet on monoaminergic content (Wurtman and Fernstrom, 1975), with high-carbohydrate meals causing increased tryptophan and 5-HT contents in

Table 3
Demographic and biochemical parameters of subjects included in the RCG study. All data are presented as mean ± SD. Significant differences after post-hoc correction for multiple comparisons ($P < 0.05$) are indicated by superscript letters a, b, c, d, e, f, g, h, i, and j for differences between C1 and C2, C1 and C3, C1 and C4, C1 and C5, C2 and C3, C2 and C4, C2 and C5, C3 and C4, C3 and C5, and, C4 and C5, respectively. Statistically significant differences after independent samples T-tests between the non-AD/CONTR and the AD group, or mixed model ANOVA, are depicted in the rightmost column. In case of 5-HIAA, the effect of CSF subsample is first represented, followed by the statistical significance of the interaction effect between CSF fraction and diagnostic class. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; AD: Alzheimer's disease; C1-5: five consecutive CSF fractions containing 4.5 mL, 1.5 mL, 1.5 mL, 4.5 mL and 4.5 mL, respectively; CONTR: control; CSF: cerebrospinal fluid; HVA: homovanillic acid; MHPG: 3-methoxy-4-hydroxyphenylglycol; RCG: rostrocaudal concentration gradient.

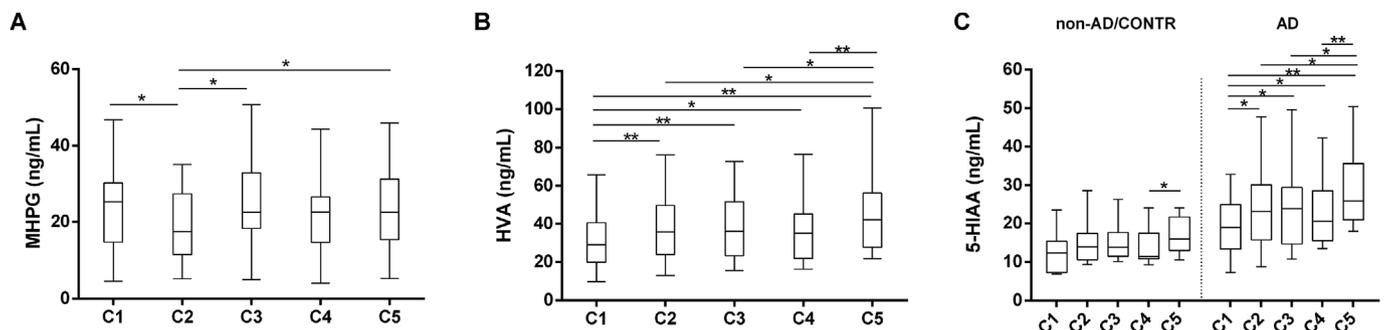| Parameter		Non-AD/CONTR	AD	Test statistic
Age (years)		68.8 ± 12.0	73.3 ± 4.7	t(11.70) = −1.11 $P > 0.05$
Gender (male/female)		5/5	5/5	$\chi^2 = 0$ $P > 0.05$
Storage time (months)		47.3 ± 3.0	47.1 ± 3.6	t(18.00) = 0.13 $P > 0.05$
CSF MHPG (ng/mL)	C1	23.8 ± 11.5[a]		F (2.66, 47.88) = 8.67 $P < 0.001$
	C2	19.1 ± 9.3[a,e,g]		
	C3	24.8 ± 12.0[e]		
	C4	22.0 ± 10.5		
	C5	23.9 ± 11.2[g]		
CSF HVA (ng/mL)	C1	30.8 ± 13.9[a,b,c,d]		F (2.32, 41.71) = 29.85 $P < 0.001$
	C2	37.5 ± 16.4[a,g]		
	C3	38.0 ± 15.9[b,i]		
	C4	35.2 ± 15.0[c,j]		
	C5	44.3 ± 18.9[d,g,i,j]		
CSF 5-HIAA (ng/mL)	C1	12.6 ± 5.1	19.2 ± 8.2[a,b,c,d]	F (2.56, 45.99) = 24.97 $P < 0.001$
	C2	15.0 ± 5.8	23.9 ± 11.3[a,g]	
	C3	15.3 ± 5.1	24.5 ± 11.4[b,i]	F (2.56, 45.99) = 3.81 $P < 0.05$
	C4	13.9 ± 4.7[j]	23.0 ± 9.0[c,j]	
	C5	16.8 ± 4.7[j]	28.8 ± 10.2[d,e,f,j]	

Fig. 3. Concentrations of MHPG, HVA and 5-HIAA in each CSF fraction (panels A, B, and C, respectively). In A and B, the combined results of the non-AD/CONTR group and the AD group are displayed, since no interaction effect was found for either MHPG or HVA. In case of 5-HIAA, the results are depicted separately for each diagnostic category. Statistically significant post-hoc differences between CSF fractions after Bonferroni correction, are indicated by one ($P < 0.05$) or two ($P < 0.001$) asterisks. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; AD: Alzheimer's disease; CONTR: control; CSF: cerebrospinal fluid; HVA: homovanillic acid; MHPG: 3-methoxy-4-hydroxyphenylglycol.

the brain. In addition, levels of DA and (N)A increase in response to meals consisting of 40% protein. Since we did not measure brain monoamine levels and since we only found evidence of a circadian rhythm in metabolites of these compounds, it is difficult to determine whether food consumption affected our findings. Nevertheless, the observed similarities in circadian rhythm of melatonin, a metabolite of 5-HT, and HVA, are not entirely consistent with a dietary influence on these molecules. Since subjects participating in this study received standardized meals mainly consisting of carbohydrates, the increase in melatonin could be foreseen. The surge of HVA at the same time point, however, is contradictory with the effect of high-carbohydrate meals. In addition, three surges in melatonin, one after each meal of the day, would also be expected if diet had truly influenced our observations.

Altogether, we propose to sample plasma and CSF at standardized times when the variation in monoamine/metabolite levels is lowest. According to our results, this would be in the early afternoon (12:00–15:00 h).

4.2. Rostrocaudal concentration gradient

As expected from previous reports, no RCG was observed for CSF MHPG, while HVA and 5-HIAA did show increasing levels in consecutive CSF fractions. Since MHPG readily passes the blood-brain barrier (Kessler et al., 1976), as well as the blood-CSF barrier (Kopin et al., 1983), CSF MHPG levels might not correlate with central noradrenergic metabolism (Kessler et al., 1976), possibly because of constant inflow from the blood compartment (Faull et al., 1990; Sharma et al., 1994). Moreover, HVA and 5-HIAA are removed from the CSF by diffusion, bulk flow of CSF and active transport mechanisms (reviewed in Spector et al., 2015). This difference in clearance mechanisms between CSF MHPG, HVA and 5-HIAA, might additionally explain the observed differences in concentration gradient of these compounds. Furthermore, a decrease in the C4 fraction was observed for HVA, 5-HIAA and MHPG, as well as a reduction of MHPG levels in the C2 fraction. It should be noted that the C2 fraction of each study subject had already been used for determination of the standard AD biomarker panel, and that CSF MHPG levels, contrary to HVA and 5-HIAA concentrations, were found to decrease even after a single freeze-thaw cycle (Willemse et al., unpublished results). Nevertheless, it remains unclear why CSF MHPG, HVA and 5-HIAA levels were reduced in the C4 fraction. In most other studies reporting the existence of a RCG in CSF HVA and 5-HIAA concentrations, a larger total volume of CSF was collected (Blennow et al., 1993; Malm et al., 1994; Sjöström et al., 1975). Thus, as the decrease in CSF analyte levels occurs after the first 7.5 mL in our study, it is possible that this reflects normal biological variation, of which the effect could have been less pronounced if larger, and most importantly, more fractions had been collected. This might also account for the shallow slope observed for all analyzed compounds in Fig. 3. Generally, our results

suggest that the presence of a RCG of monoaminergic metabolites such as HVA and 5-HIAA, might indeed influence the comparison of these compounds between distinct factorial groups. We therefore suggest that investigators analyze the first CSF fraction from all study participants, so that the possible effect of the RCG is avoided.

Lastly, we observed consistently higher CSF 5-HIAA levels in the AD group versus non-AD/control participants. Different factors might account for this apparent discrepancy with the existing literature, in which predominantly lower or unchanged CSF 5-HIAA levels were noted in AD patients versus controls (Blennow et al., 1992; Janssens et al., 2018; Sjögren et al., 1998), and in which a negative correlation was found between AD severity and brain 5-HT and 5-HIAA levels (Rodriguez et al., 2012; Vermeiren et al., 2014). The most important factor is probably the presence of non-AD dementia types, which might also display disturbances in monoamine content, in the 'control' group. In addition, the individual dementia stage of the patients at the moment of CSF collection, might also have influenced our findings.

4.3. Strengths, limitations and relevance

All analyses for the study of circadian rhythm were performed on a valuable and extensive dataset incorporating paired CSF and plasma samples derived from eight healthy volunteers, during a 30-h period. It should be noted, however, that only seven subjects completed the whole protocol, possibly decreasing statistical power. Samples were thawed twice beforehand, which might have impacted our results as freeze/thaw cycles could affect the stability of monoaminergic compounds. However, it has been demonstrated that CSF HVA and 5-HIAA remain relatively stable even after six freeze-thaw cycles of variable duration (Strawn et al., 2001; Willemse et al., unpublished results). In addition, plasma melatonin levels were previously found to be unchanged after three freeze/thaw cycles (Graham et al., 1998). The results of the linear mixed model analysis were concordant with the circadian rhythm findings, illustrated by the fact that the individual acrophases corresponded with sampling time 14, which was the only time point at which plasma melatonin levels differed significantly compared to almost all other time points. Therefore, another strength of this study can be found in the relatively accurate estimation of diurnal melatonin rhythm. On the one hand, the long sampling intervals during the night might have hampered an even more meticulous evaluation of the circadian rhythm of all compounds, as the acrophases of all diurnal compounds occurred during these four-hour intervals. On the other hand, the circadian rhythm might have been disturbed if the subjects were woken up more frequently during the night for sampling purposes. Furthermore, as the circadian rhythm of melatonin is widely accepted to occur around 02:00–04:00 h (Cajochen et al., 2003; Zeitzer et al., 2007; Zhdanova et al., 1998; Zisapel, 2018), our results corroborated these previous findings. Thus, melatonin could be considered a positive

control compound in this circadian rhythm study.

Concerning the investigation of the RCG, the use of CSF samples derived from subjects who underwent neuropsychological assessment and CSF biomarker analysis, increased the reliability of our results. Moreover, storage- and handling conditions were similar across all samples, thereby minimally affecting differences between groups or CSF fractions. However, the disease state of our study subjects has not been confirmed neuropathologically, and the sample size used for this investigation was rather small, so that the effect of a possible RCG on monoaminergic metabolites might have been more pronounced if more subjects were included in the analysis.

This work focuses on the methodological aspects of neurochemical (biomarker) studies investigating monoamines and/or their metabolites. These compounds are especially of interest since they were previously found to be altered in several forms of dementia (Aerts et al., 2011; Herbert et al., 2014; Janssens et al., 2018; Parnetti et al., 1992; Sjögren et al., 1998; Vermeiren et al., 2016) and since currently, most of the available treatment options are associated with a lack of efficiency and/or important side effects. Therefore, the assessment of alterations in these compounds should be free of misinterpretation and/or bias due to the circadian rhythm and the RCG, so that monoamines/metabolites can be identified as reliable biomarkers and/or therapeutic targets in dementia.

5. Conclusions

In general, pre-analytical factors should be carefully considered before the onset of studies investigating (monoaminergic) compounds in biological fluids. Despite relatively small study populations in this work, we found that plasma HVA levels vary according to a circadian rhythm similar to that of melatonin, which could be explained by a shared control mechanism in the SCN. Our results indicated that plasma and CSF should ideally be sampled in the early afternoon for the analysis of monoamines and their metabolites. Lastly, since a RCG was observed for CSF HVA and 5-HIAA, it is advisable to standardize the number and volume of CSF samples, so that comparable biological specimens can be analyzed in future studies. To avoid confounding effects of the RCG, we propose to analyze the first 1.5 mL of CSF collected in all study participants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuint.2019.04.015.

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