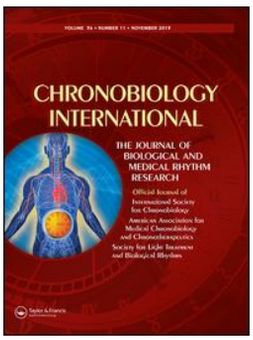

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The Possible Role of Endozepines in Sleep Regulation and Biomarker of Process S of the Borbély Sleep Model

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ABSTRACT

The well-known Two-Process Model of Sleep Regulation describes the integration of the circadian rhythm of arousal and sleep – Process C, and the homeostatic pressure to sleep – Process S. Presently, the known biological markers for Process C are melatonin and core body temperature; whereas, for Process S, there is no biological marker except that of aspects of the electroencephalogram (EEG). Endozepines are a class of endogenous compounds that act like benzodiazepines (BZ), i.e., serving as ligands for the BZ binding sites on $GABA_A$ receptors. Not much is known about the role of endozepines, in particular non-peptide endozepines, in the sleep field except very few reports about high concentrations observed in endozepine stupor, a rare phenomenon of idiopathic recurring stupor. We focused on hypoxanthine and thromboxane A2, which are considered to have endozepine function. This study aimed to examine the effect of 24 h of acute sleep deprivation on blood levels of hypoxanthine and thromboxane A2 of healthy subjects without sleep problems or disorders. The results showed a significant decrease of both compounds in the morning after sleep deprivation in comparison to the unrestricted normal sleep condition, thereby suggesting that these endozepines are secreted regularly while asleep, and, thus, are necessary for the sleep process. This study is the first to suggest a connection between specific biological markers – endozepines and Process S – in the Two-Process Model of Sleep Regulation and, furthermore, it sheds light on the possible role of endozepines in sleepiness and fatigue.

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Introduction

Almost four decades ago, the Two-Process Model of Sleep Regulation was proposed by Borbély (1982). Since then it has a significant impact on sleep research, and the research around the model continues. Validation of the model was made, and the clinical use for evaluating work/rest schedules is based on it (Achermann and Borbély 2003; Akerstedt and Folkard 1995; Daan et al. 1984). The Two-Process Model assumes that the tendency for sleep and sleepiness is managed by the interaction of Process S – a homeostatic pressure for sleep, and Process C – a circadian rhythm of arousal. As hours of wakefulness accumulate, the pressure to sleep increases (represented by Process S) and decreases while asleep. Process C that promotes sleep at night and alertness during the day is affected by a circadian pacemaker, the suprachiasmatic nucleus. The known biological markers of Process C are core body temperature and melatonin secretion. Measurement of Process S relies on the electroencephalogram (EEG) of slow-wave activity (SWA), which is used as a marker through sleep time, and theta activity during wake time (Daan et al. 1984). There is no biological compound as yet found that is a biomarker for Process S. During sleep deprivation, there is no significant change in the amplitude of Process C, but it may undergo phase shift like other circadian markers that occurs with transmeridian travel and shift work (Borbély et al. 2016). In contrast, Process S is known to be affected by sleep deprivation, as the hours of wakefulness increases (Figure 1) (Patanaik 2015).

Since the publication of the Two-Process Model, several additive and interactive models have been proposed (Achermann and Borbély 2003; Borbély and Achermann 1999; Dijk and Archer 2010; Folkard et al. 1999; Jewett and Kronauer 1999). One major suggestion is that there is another process that needs to be considered. Such third process, sleep inertia or Process W, reflects the fact that people typically feel sleepy upon awakening, and, therefore, it influences sleepiness and wakefulness together with Processes C and S (Hilditch and Mchill 2019). The proposal that there is an interaction between

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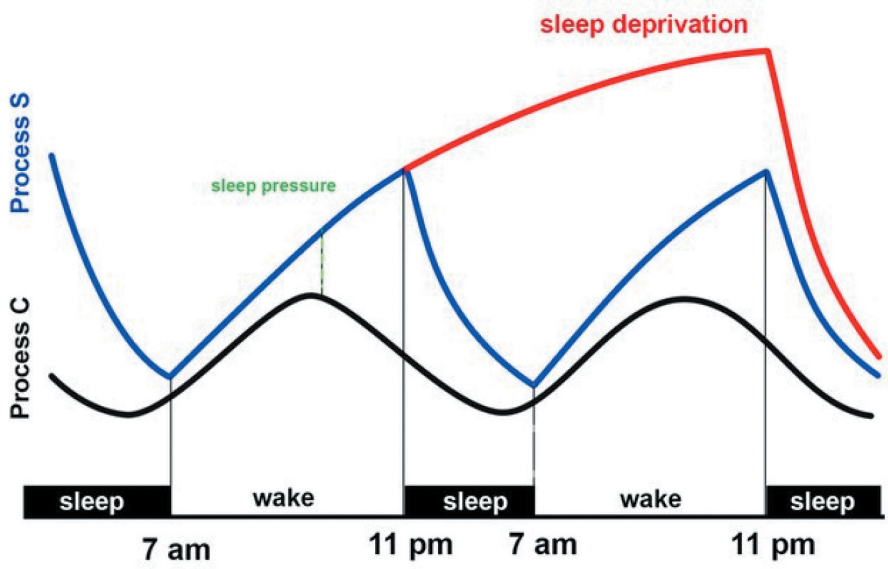

Figure 1. The two-process model of sleep regulation. Process C represents the circadian rhythm of arousal with no influence by sleep deprivation (black). Process S represents the homeostatic pressure for sleep (blue) and increases as long there is no sleep (red). Quantification of sleep pressure is determined by the difference between the two processes. Patanaik (2015).

the three processes raised the question about the relationship between the components of the model and the thermoregulatory system. Kräuchi et al. (2006) found that there is an influence of the thermoregulatory system on Processes C and W that is independent of Process S.

Benzodiazepines (BZ) is a class of medications that have been developed for the treatment of sleep disorders and anxiety. During the last 60 y, thousands of BZs have been developed, and many of them are used clinically. Studies over the years have identified two classes of BZ receptors/binding sites. One class in the central nervous system (CNS) includes γ-aminobutyric acid type A receptor ($GABA_AR$), and the other class in the peripheral tissues is known as translocator protein (TSPO). When these receptors are activated, they appear to have a calming effect; thus, they are used as target sites for anxiety and insomnia medications (Griffin et al. 2013). A group of endogenous ligands for the BZ binding sites has been identified and named "endozepines" (Rothstein et al. 1992). It is noteworthy that a unique sleep disorder – idiopathic recurring stupor (IRS) – termed "Endozepine Stupor" is a syndrome of spontaneous coma characterized by fast EEG activity. Patients with this syndrome when undergoing screening were found to have a high level of endozepines, in the absence of exogenous benzodiazepine administration, and that the stupor could be reversed by flumazenil, a benzodiazepine antagonist (Lugaresi et al. 1998). Some endogenous compounds that may serve as endozepines have been studied and reported (Farzampour et al. 2015). In the study reported herein, we focused on hypoxanthine and thromboxane A2, which were found to be endogenous ligands for BZ binding sites (Asano and Spector 1979; Schwartz-Bloom et al. 1996).

Sleep deprivation is used as a method in sleep research, and it has been shown to exert different effects on the Two-Processes Model of Sleep Regulation. Most investigations that used this method focused on known markers of the model, such as melatonin levels, thermoregulatory system, EEG changes, etc., and some focused on behavioral changes, like the anti-depressive effects of sleep deprivation by its influence on the sleep regulatory system (Achermann 2004; Borbély et al. 1981, 2016; Deboer et al. 2007). The association between endozepines and the Two-Process Model of Sleep regulation has never been reported. Taken together with the knowledge about the natural function of endozepines as sleep mediators, the aim of the present study was to assess changes in endozepine levels before and after acute sleep deprivation of healthy sleepers for a possible link to Process S, as this process is known to be affected by sleep deprivation. We hypothesized that significant change in hypoxanthine and thromboxane A2 levels before and after sleep deprivation suggests their involvement in sleep regulation. Specifically, if endozepines are truly involved in sleep regulation and their levels increase while sleeping, we suspect that in the absence of sleep there will be a decrease in their secretion as the body's "fight" against the urge to sleep.

Methods

Participants

The study involved 30 healthy young subjects (15 males, 15 females) aged between 24 and 30 y (27 ± 1.35 y; mean ± SD) with regular sleep patterns, as measured by the Pittsburgh Sleep Quality Index (PSQI; a score of <5 for inclusion), and a normative sleep-wake cycle, as indicated by the Morningness-Eveningness Questionnaire (MEQ; score between 42 and 58 for inclusion). Sleep quality and continuity were measured for six nights prior to sleep deprivation using wrist actigraphy (Respironics Model II, Philips, Inc. USA). Subject inclusion criteria were 6 to 8 h of nightly sleep, regular sleep-wake pattern, and no sleep-wake schedule problems. Subject exclusion criteria were BMI <18 or >25, any history of medical, gastrointestinal, neurological, psychiatric, or known sleep disorders, and regular consumption of medications (except female participants who took contraceptives). All participants signed informed consent prior to participation in the study that was approved by the Helsinki Committee of Assuta Medical Center, Israel, and the study was performed according to international ethical standards for biological rhythm research studies (Portaluppi et al. 2010).

In-laboratory session

First, subjects were requested to maintain a regular sleep/wake schedule (6–8 h sleep/night), which was confirmed by wrist actigraphy, for six nights prior to the experimental phase. The normal sleep control condition took place in each of the subject's home environment, i.e., bedroom. Normal duration and quality of sleep during the control (baseline night) were verified by wrist actigraphy. On day 7 of the study, subjects arrived at approximately 08:00 h, immediately after awakening from their normal sleep at home, to our Applied Chronobiology Research Institute for blood sampling (Baseline blood sample), completion of the Karolinska Sleepiness Scale (KSS) and Epworth Sleepiness Scale (ESS) questionnaires, and for conduct of the acute sleep deprivation (ASD) condition of 24 h duration.

The 24 h sleep deprivation condition was supervised by the researchers. Subjects were not allowed to consume caffeine or other stimulants during the 24 h ASD conditions. On day 8, at the conclusion of the ASD condition, a second blood sample (ASD blood sample) was collected from each of the subjects at the same time of the Baseline blood sample, at approximately 08:00 h.

Samples preparation

Blood samples for metabolomic analysis were collected into EDTA tubes and separated by centrifugation. Plasma fractions were stored at −28°C until extraction for Ultra-High-Performance Liquid Chromatography (UHPLC) and Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) analysis. The samples were thawed on ice before extraction using 300 µL plasma and 1200 µL solvent (methanol) with a 2 min vortex step before centrifugation at 13,000 rpm and 4°c for 15 min. Extracts were transferred into new Eppendorf tubes followed by evaporation with nitrogen, thawing with 150 µL of 50% methanol, and centrifuging at 13,000 rpm and 4°c for 10 min. A 120 µL of the extracts was transferred into 1.5 ml vials with insert and short thread cups (LLG Labware) suitable for UHPLC and LC-MS/MS. Quality control (QC) samples were prepared by mixing 20 µL from each extract and a blank vial was made with methanol alone.

UHPLC analysis

Untargeted metabolomic analysis was carried out first on a UHPLC connected to a photodiode array detector (Dionex Ultimate 3000), with a reverse-phase column (ZORBAX Eclipse Plus C18, 100*3.0 mm, 1.8 µm) followed by LC-MS/MS analysis. A 5 µL of each of the extracted solutions was injected into the UHPLC. The mobile phase consisted of (A) Deuterium-Depleted Water (DDW) with 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The 36 min elution gradient comprised the following: started with 98% A and increased to 30% B in 4 min, then increased to 40% B in 1 min and kept isocratic at 40% B for another 3 min. The gradient increased to 50% in 6 min, and increased to 55% in 4 min, and finally increased to 95% in 5 min and kept isocratic for 7 min. Phase A was returned to 98% A 3 min, and the column was allowed to equilibrate at 98% A for 3 min before the next injection. The flow rate was 0.4 mL/min. QC samples were run approximately one in every eight injections to assess the analytical variability.

LC–MS/MS analysis

LC–MS/MS analysis was performed with a Heated Electrospray ionization (HESI-II) source connected to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer Thermo Scientific™. ESI capillary voltage was set to 3500 V, capillary temperature to

350°c, gas temperature to 350°c, and gas flow to 10 mL/min. The mass spectra (m/z 67–1000) were acquired in positive-ion mode. Tandem MS (MS/MS) analysis was used to identify and separate ions that had similar m/z ratios after analysis of the first mass spectrometer.

Data preprocessing and statistical analysis

Normalization of determined peaks and areas under the peaks was accomplished using the QC samples that were injected during the sequence after every eight samples. Peak determination and integration of peak area were performed with Compound Discoverer™ software (Thermo Xcalibur, Version 3.1.0.305). Compounds were identified by the Compound Discoverer™ software integration with ChemSpider database. Compounds that went through MS/MS were identified using the mzCloud database. The obtained peaks of each compound were analyzed using the two-tailed t-test (Compound Discoverer™) to examine the effect of sleep deprivation by comparison between the mean of peaks' area values indicative of the hypoxanthine and thromboxane A2 levels of the Baseline blood samples versus levels of the ASD blood samples. Values of $p < .01$ were considered significant.

Results

Using untargeted LC-MS/MS analysis, hypoxanthine was identified by the detection level of 90% in all samples. The mean area of the peaks of both the Baseline and ASD condition blood samples was calculated; one outlier (value >3-fold beyond the standard variation of the mean) from the ASD Group was excluded. The average blood level of hypoxanthine after conclusion of the ASD condition was decreased by 0.49-fold compared to that of the normal sleep condition (n = 59, $p < .00001$ [Figure 2]).

Thromboxane A2 was identified by untargeted LC-MS analysis. After calculation of the mean area of the peaks, two outliers of the ASD condition and three of the Baseline condition were excluded. The average blood level of thromboxane A2 following the ASD condition was lower by 0.36-fold compared to that of the Baseline condition (n = 55, $p < .005$ [Figure 3]).

Discussion

The Two-Process Model of Sleep Regulation has been used for research and as a clinical approach for years. The model describes the integration of the circadian rhythm of arousal, Process C, and the homeostatic pressure to sleep, Process S. To the best of our knowledge,

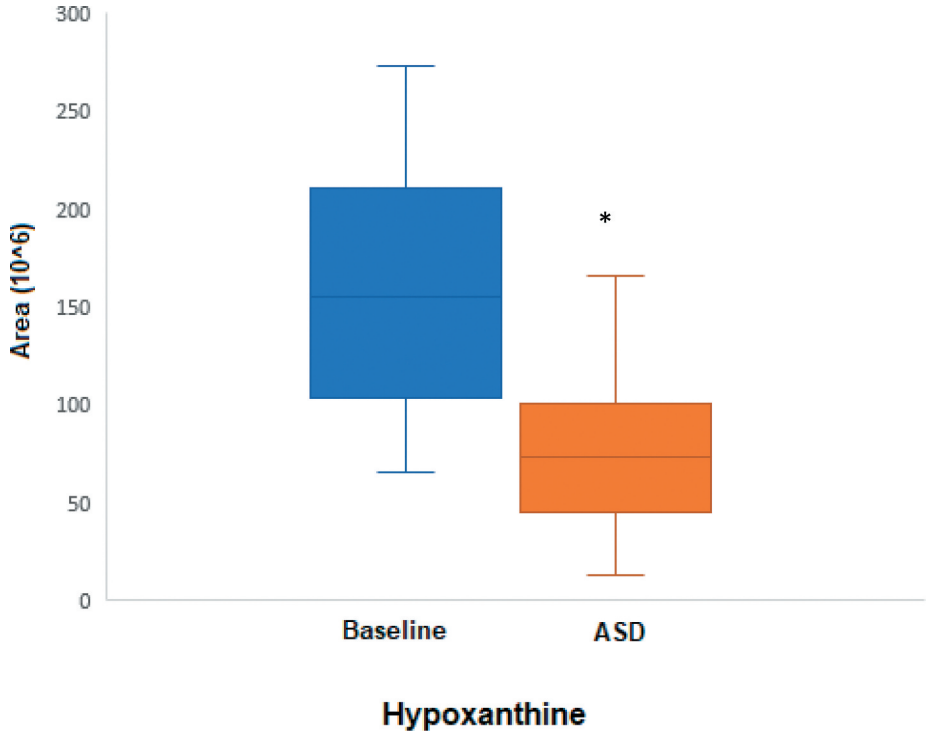

Figure 2. Change in hypoxanthine levels. Differences in the mean area of the hypoxanthine peak values of the Baseline normal sleep condition and acute sleep deprivation (ASD) condition after normalization of values. Data represented as max, min, and median values of the peak areas for the hypoxanthine peak of 30 baseline blood samples (blue) and 29 ASD blood samples (orange). **P<.00001.*

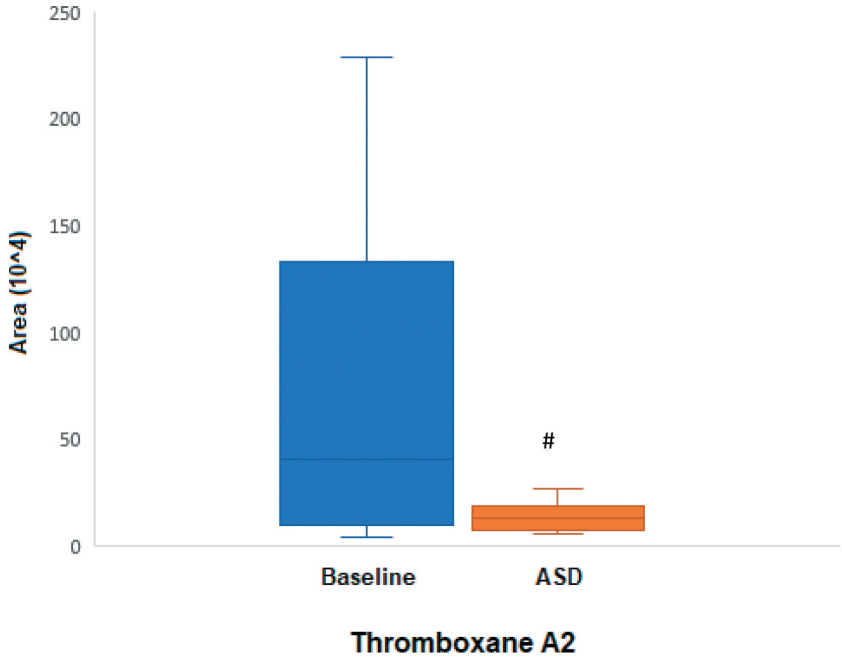

Figure 3. Change in thromboxane A2 levels. Differences in mean area of the thromboxane A2 peak values between the Baseline normal sleep condition and acute sleep deprivation (ASD) condition after normalization of values. Data represented as max, min, and median values of peak areas of 30 baseline blood samples (blue) and 29 ASD blood samples (orange). *#P<.005*.

there is no known biological compound that explains Process S as melatonin and core body temperature (CBT) explains Process C. The only known measurement for Process S is the EEG and compared to the convenience of blood samples it is not an easy marker to measure. Furthermore, while Process S is known to be affected by sleep deprivation as the sleep pressure increases, Process C in contrast does not increase or decrease, although its phasing can be shifted with alteration in the sleep/wake routine (Borbély 1982; Daan et al. 1984). Endozepines are endogenous compounds that act like benzodiazepines, i.e., serving as ligands for the BZ binding sites on $GABA_A$ receptors. This binding to $GABA_AR$ leads to sedative and sleep-inducing effects, thus BZs are used as common anxiety and insomnia drugs (Farzampour et al. 2015; Tonon et al. 2020).

There has been relatively little investigation in the field of sleep medicine of endozepines, in particular non-peptide endozepines, and their functions, although the knowledge we do have may suggest they have an impact on sleep in daily life. Furthermore, currently, the main reported relationship between endozepines and sleep has been conceptualized in the context of endozepine stupor, a phenomenon of idiopathic recurring stupor (Cortelli et al. 2005). Here we present novel insight into the involvement of endozepines in sleep and the 24 h sleep/wake cycle.

As endozepines have a connection to sleep through the receptors as previously discussed herein, we hypothesized that after the absence of sleep there would be a change in their levels that can come together with the change in Process S while sleep deprived. We focused on two non-peptide compounds that have been found to act as endozepines – hypoxanthine and thromboxane A2 (Asano and Spector 1979; Schwartz-Bloom et al. 1996). This study aimed to assess the effect of acute sleep deprivation of 24 h on levels of hypoxanthine and thromboxane A2 measured in plasma collected in the morning after normal nighttime sleep – in the absence of ASD – and after ASD from the same 30 healthy subjects. Our results showed a significant decrease in the two endozepines of hypoxanthine and thromboxane A2 in the morning after sleep deprivation in comparison to the morning after usual sufficient sleep.

These results suggest that endozepines are secreted during sleep and, therefore, their levels are lower after acute sleep deprivation. This is in contrast to melatonin and CBT, in which each expresses its circadian rhythm separately from the presence or absence of sleep. When there is a shift in Process C, as a result of shift work or jet lag, there is also a shift in its markers as they synergically vary with Process C, but independently from Process S. It is unclear whether and how the two processes influence each other directly, and future studies with longer sleep deprivation may suggest some answers (Deboer 2018).

This study is the first to suggest a connection between specific endozepines and Process S in the Two-Process

Model of Sleep Regulation. This connection may be useful to explain Process S in physiologically or theoretically manners in future investigations. The report of our significant results herein suggests the importance of endozepines in sleep regulation, thus promoting more studies that focus on endozepines and their role in sleep. Moreover, it is known that sleep deprivation exerts antidepressant effects, but the mechanisms of this impact remain unclear (Dallaspezia and Benedetti 2015). If endozepines are truly involved in sleep regulation, it is possible that they also may play a role in depression and anxiety, an issue that should be focused on in future studies.

As this study presents preliminary results, future experiments are needed to examine the role of endozepines in the normative sleep-wake cycle by monitoring their levels constantly throughout a 24 to 48 h span. Our assumption that endozepines are secreted while asleep can be explained by the presented results, but to establish this assumption, it is necessary to scan their levels in subjects while they sleep. Additional future studies should evaluate the role of endozepines in the induction of normal daytime sleepiness as well as pathological sleepiness, such as idiopathic hypersomnolence or narcolepsy. As described in the Introduction section of this article, there are more suspected compounds that serve as endozepines (Tonon et al. 2020). The Present study focused on hypoxanthine and thromboxane A2, and it is important to examine more compounds that may be endozepines in similar future studies. In addition to supporting the Two-Process Model of Sleep Regulation and providing more biological enlightenment with regard to sleep homeostasis, our findings possibly have future implications for their clinical use to diagnose and even treat certain sleep problems and disorders.

Disclosure statement

Ms. Simona Sher, Dr. Amit Green, Professor Soliman Khatib, and Professor Yaron Dagan declare that they have no conflict of interest. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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