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Exploring Hypoxanthine Endozepine as a Potential Biomarker for Daytime Sleepiness

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Abstract

Benzodiazepines (BZs) interact with GABAA receptors, influencing neurotransmission and contributing to sedation and sleep regulation. Endogenous ligands for BZs, known as endozepines, have been implicated in sleep disorders, including recurrent stupor characterized by sudden sleep attacks. The current study investigates variations in endozepine levels, focusing on hypoxanthine as an endozepine, across diurnal and nocturnal periods in relation to daytime sleepiness. Eighteen subjects underwent overnight Polysomnography (PSG) followed by Multiple Sleep Latency Tests (MSLT). Blood samples collected at multiple time points were analyzed using LC-MS/MS for hypoxanthine concentrations. Subjects were categorized into "Sleepy" (mean sleep latency ≤ 9 minutes, consistent with community-based sleepiness thresholds) and "Not sleepy" groups based on MSLT results. The "sleepy" group exhibited significantly lower morning (06:00 h) hypoxanthine levels compared to the "not sleepy" group, but higher levels of hypoxanthine in the late morning (10:00 h) compared to the "not sleepy" group. Notably, the "sleepy" group displayed longer total sleep time during MSLT naps, higher sleep efficiency, a greater percentage of REM sleep, and a higher number of REM episodes in MSLT, suggesting a correlation between increased REM sleep and decreased hypoxanthine levels. These findings suggest a potential biomarker role for hypoxanthine in assessing daytime sleepiness and underscore the complex interplay between endozepines, REM sleep, and sleepiness disorders.

Keywords: Endozepine; Hypoxanthine; REM; GABAA; Sleepiness; MSLT

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Introduction

Benzodiazepines (BZs) were first introduced in the 1960s and have remained among the most prescribed drugs, acting as sedatives, hypnotics, anxiolytics, anticonvulsants, and muscle relaxants [1,2]. The molecular mechanism of BZ activity was first elucidated after the discovery of the connection between BZs and GABAA (Gamma-Aminobutyric Acid Type A) receptors. GABA is an amino acid that serves as the primary inhibitory neurotransmitter in the brain and central nervous system [3,4]. This connection demonstrated that BZ receptors contain binding sites for both GABA and BZs, indicating that BZs and GABA are linked to the same receptor complex. When these receptors are activated, they contribute to feelings of fatigue and drowsiness, making them useful as sedatives and sleep aids. The discovery of the binding site for BZs suggested that the brain produces endogenous ligands for BZs [5].

There is a syndrome called recurrent stupor that occurs in patients with sleep attacks. Patients experiencing this phenomenon fall into a deep, prolonged sleep with rapid EEG activity, and the attacks can occur at any time of day. High levels of endogenous ligands for BZs have been measured in the plasma of these patients during attacks. The drug flumazenil, a BZ antagonist, has been used as a therapeutic option during attacks, leading to the immediate awakening of patients. Without the drug, patients typically awaken spontaneously after hours or days [6,7].

Endogenous ligands for BZs are also called endozepines, and several substances suspected to be endozepines exist. One is the Diazepam Binding Inhibitor Protein (DBI), which is linked to the GABAA receptor. The name DBI reflects the protein's role in interacting with diazepam, a type of benzodiazepine, and modulates its effects [8].

Endozepines can be classified into two categories: peptide endozepines (such as DBI and its processing products) and non-peptide compounds that bind to benzodiazepine recognition sites on the GABAA receptor. Hypoxanthine, a purine derivative and metabolite of adenosine metabolism, belongs to the latter category. While structurally distinct from classical benzodiazepines, hypoxanthine demonstrates binding affinity for the GABAA receptor complex [9], qualifying it as a non-peptide endozepine with endogenous regulatory potential.

Recent research has examined the relationship between endozepines and sleep homeostasis. In one study, substances suspected to be endozepines were tested after acute sleep deprivation. Blood samples were collected in the morning after sufficient sleep and again following 24 hours of sleep deprivation. Hypoxanthine levels were higher following sleep deprivation compared to baseline, suggesting hypoxanthine as a potential biomarker for homeostatic sleep pressure (Process) in Borbély's two-process model of sleep regulation [10].

Benzodiazepine drugs are known to have a somnolent effect; however, research in this field has not yet investigated the relationship between endozepines and daytime sleepiness.

In this study, we explore the variations in endozepine concentrations in the blood over both nocturnal and diurnal periods, aiming to establish a potential relationship between these levels and daytime sleepiness as measured by sleep tests. Thus, we aim to determine whether measuring endozepine levels could serve as an objective biological marker for fatigue and sleepiness. We suggest that daytime sleepiness in individuals might be associated with variations in hypoxanthine levels.

Methods

Subjects

The study involved 18 subjects (10 males, 8 females) aged 20-64 years. Five of the subjects were patients who complained of daytime sleepiness and were referred by physicians to a sleep laboratory for evaluation. The remaining participants responded to a clinical study advertisement requesting individuals suffering from daytime sleepiness to register for the study. Only those who completed the Epworth Sleepiness Scale (ESS) and received a minimum score of 11 were included in the research. Exclusion criteria were any history of medical, gastrointestinal, neurological, psychiatric, or known sleep disorders, and regular consumption of medications (except female subjects who took contraceptives). All subjects signed informed consent prior to participation in the study, which was approved by the Helsinki Committee of Assuta Medical Center.

This is a small study, and the sample size was limited to 18 subjects due to the intensity of the protocol and practical constraints of in-lab monitoring (overnight PSG, repeated blood sampling, and serial MSLT assessments). Despite the small sample, the design allowed for detailed phenotyping and repeated measures in a controlled environment, which increases the reliability and specificity of the observed findings.

Study Procedure

Pre-study protocol

Participants were instructed to maintain regular sleep-wake schedules for at least one week prior to the study night. Participants reported their prior-night sleep duration and compliance with pre-study instructions.

The subjects arrived in the evening and underwent overnight Polysomnography (PSG). The following day, they completed a Multiple Sleep Latency Test (MSLT), which is considered the gold standard objective measure of daytime sleepiness [11,12]. The MSLT consisted of five 20-minute nap opportunities at 08:00, 10:00, 12:00, 14:00, and 16:00h. Each nap was terminated either 15 minutes after sleep onset or after 20 minutes if no sleep occurred. Sleep-onset REM periods (SOREMs) were recorded when REM sleep occurred within 15 minutes of sleep onset. Testing was conducted in a darkened bedroom with ambient light less than 0.1 lux. Participants remained in a semi-recumbent position for all naps.

The subjects were set up for sleep channel monitoring by a certified sleep technician, which included Electromyography (EMG), Electroencephalography (EEG), Electrooculography (EOG), Electrocardiography (ECG), respiratory, and position channels. Additionally, they were fitted with venous blood sampling catheters to collect blood samples during the research period. Blood samples were taken upon catheter insertion at 22:00, 03:00, and 06:00h and before each nap during the MSLT. Manual scoring of all PSG and MSLT naps was performed by certified sleep technologists according to American Academy of Sleep Medicine (AASM) 2017 guidelines [13].

Blood samples preparation

Blood samples were collected in an anticoagulant-free tube, incubated for 30 minutes, and separated by centrifugation. The separated material was transferred to a 2 mL Eppendorf tube and frozen at -20˚C. For targeted metabolomic analysis, 500 µL of the serum was mixed with 1000 µL of methanol, vortexed for 3 minutes, and centrifuged (10 min, 12,000 rpm, 4˚C). The supernatant evaporated under a nitrogen stream, then lyophilized until fully dried. The resultant pellet was reconstituted in 500 µL of 50% methanol in DDW, centrifuged (10 min, 12,000 rpm, 4˚C), and 200 µL was transferred to HPLC vials. Blank vials consisted of 50% MeOH in DDW. The QC samples were prepared by mixing 20 µL of each sample. Blank and QC samples were injected first in the sequence, after each set of 10 samples, and at the end of the sequence. All vials were stored at -20°C until analysis in the LC-MS/MS.

HPLC analysis

Untargeted and targeted analysis were carried out with the same method as follows. The extract (5 µL) was injected into a UHPLC connected to a photodiode array detector (Dionex Ultimate 3000), with a reverse-phase column (ACE C18, 100 mm × 3.0 mm, 1.7 µm, Avantor, PA, USA). The mobile phase consisted of (A) DDW with 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The gradient started with 2% B then increased to 30% B in 4 minutes, then increased to 40% B in 1 minute and kept isocratic at 40% B for 3 minutes. Then increased to 50% in 6 minutes, then increased to 55% in 4 minutes and then increased to 95% B in 5 minutes and kept isocratic at 98% B for 6 minutes. Phase B returned to 5% in 2 minutes and the column was allowed to equilibrate at 5% B for 5 minutes before the next injection. The flow rate was 0.4 mL/min. Column temperature was set to 30˚C.

MS/MS analysis

Mass Spectrometry (MS) analysis was performed with 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 3900 V, capillary temperature to 350°C, gas temperature to 350°C and gas flow to 35 mL/min. The mass spectra (m/z 67-1000) were acquired in negative and positive-ion mode with high resolution (FWHM=70,000). For MS2 analysis, collision energy was set to 20, 30 and 50 eV.

Data pre-processing

Data acquisition and analysis of all spectra were conducted using Thermo Scientific™ Xcalibur™ instrument control software. Extracted ion chromatograms for figure production were generated in Xcalibur 4.0.27.19 software using a mass window of (+/−) 5 ppm. Exact m/z values for hypoxanthine used for figure creation and peak integration corresponded to theoretical [M + H] + ion m/z values 137.0458 m/z. Peak integration was performed using QNTM software after determining LOD, LOQ and linearity for each analyte.

Statistical Analysis

Data were analyzed using repeated measures ANOVA to compare hypoxanthine concentrations across time points between the "Sleepy" and "Not sleepy" groups. Subjects were categorized using a 9-minute MSLT threshold based on normative population data and community-based research on sleep-wake regulation (detailed rationale provided in Results). Independent samples t-tests were used to compare PSG and MSLT parameters (sleep efficiency, REM percentage, REM latency, and total sleep time) between groups. For the sleep stage analysis, each PSG recording was divided into two-time segments (22:00-03:00 h and 03:00-06:00 h), and comparisons of REM percentages between segments within and between groups were performed using appropriate t-tests. Statistical significance was set at $p < 0.05$ for all analyses. Data are presented as mean ± standard deviation unless otherwise specified.

Results

Based on MSLT results, mean sleep latency across all five naps was calculated for each subject. Subjects were categorized into two groups: "Sleepy" (mean sleep latency ≤ 9 minutes) and "Not sleepy" (mean sleep latency >9 minutes). This threshold was selected based on normative population data showing mean sleep latencies of 11.7 minutes in healthy adults and community-based research demonstrating that 9-minute cut-offs effectively identify individuals with physiologically meaningful alterations in sleep-wake regulation.

The 9-minute threshold was selected based on several converging lines of evidence. Meta-analysis of normative MSLT values in healthy adults demonstrates a pooled mean sleep latency of 11.7 minutes (95% CI: 10.8-12.6), with substantial individual variability [14]. Population-based studies show that mean sleep latencies around 9 minutes represent a threshold where physiologically meaningful differences in sleep-wake regulation can be detected, optimally separating individuals with increased sleep propensity from those with normal daytime alertness in community samples [15]. Community-based research examining sleepiness across the population spectrum shows that mean sleep latencies around 9 minutes identify individuals with increased daytime sleepiness who demonstrate measurable alterations in sleep-wake regulatory mechanisms [16,17]. A 9-minute threshold allows exploration of hypoxanthine as a biomarker across a broader spectrum of daytime sleepiness while maintaining clear group distinction. This approach is consistent with recent investigations of MSLT predictors and sleep-wake regulation in community samples, which examine sleepiness as a continuous physiological variable rather than strictly as a diagnostic category. Mathematical modelling of MSLT data demonstrates that sleep latency values around 9 minutes are particularly informative for understanding sleep-wake regulation, as they capture individuals at the threshold of increased sleep propensity. Therefore, the selection of a 9-minute threshold in this study facilitated the exploration of hypoxanthine as a biomarker across the sleepiness spectrum while maintaining distinction between individuals with and without increased sleep propensity.

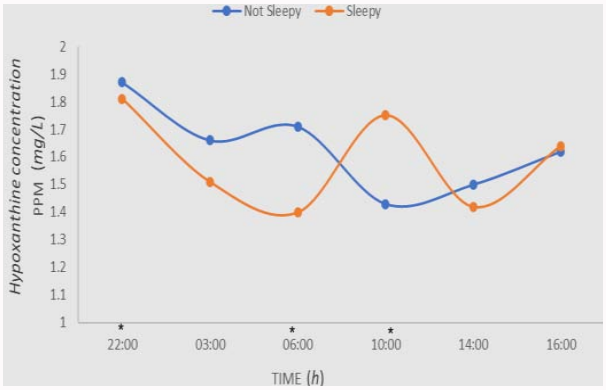

Figure 1: Temporal variation in hypoxanthine concentrations across nocturnal and diurnal periods in sleepy and non-sleepy subjects. Hypoxanthine levels were measured at six time points in subjects categorized as "Sleepy" (mean MSLT ≤ 9 minutes, orange line, n=9) and "Not sleepy" (mean MSLT>9 minutes, blue line, n=9) Asterisks indicate significant between-group differences (*p<0.05, repeated measures ANOVA) at 22:00 h, 06:00 h, and 10:00 h. The "Sleepy" group exhibited significantly lower hypoxanthine concentrations at 06:00 h (end of nocturnal sleep) but higher levels at 10:00 h (late morning), demonstrating an inverse temporal pattern compared to the "Not sleepy" group. This crossover pattern suggests differential regulation of hypoxanthine metabolism between groups across the sleep-wake cycle.

Hypoxanthine levels

The data from the LC-MS device were analyzed to compare substance levels across different time points and between the “Sleepy” and “Not sleepy” groups using repeated measures ANOVA for hypoxanthine concentrations. Substances with significant changes ($p<0.05$) were identified Table 1.

To investigate a potential circadian rhythm in hypoxanthine, mean concentrations were compared at 22:00, 03:00, 06:00, 10:00, 14:00, and 16:00h for both groups. Repeated measures ANOVA revealed significant differences in hypoxanthine concentrations between the groups at 22:00, 06:00, and 10:00h ($p<0.05$). Results showed that in the “Sleepy” group, hypoxanthine levels differed notably, with lower concentrations at 06:00h compared to the “Not sleepy” group, followed by a rise at 10:00h (Figure 1).

Sleep characteristics

PSG characteristics of the sleep and MSLT tests were analyzed to describe sleep patterns across groups Table 2. Summarizes the PSG characteristics. Independent samples t-tests revealed that the Sleepy group’s sleep was more efficient compared to the “Not sleepy” group ($p=0.05$). The “Sleepy” group showed a significantly higher percentage of REM sleep during the night compared to the “Not sleepy” group ($p=0.005$).

The concentration of hypoxanthine consistently differed between the two groups across all measurements, with the most pronounced disparity observed at 06:00 and 10:00 h. At 06:00 h, the sleepy group showed a decrease in hypoxanthine levels, which then increased by

Table 1: Hypoxanthine concentrations by time point and sleepiness level.

Time Point		Sleepy Group		Not Sleepy Group
	n	Mean ± SD (mg/L)	n	Mean ± SD (mg/L)
22:00 h (T1)*	9	1.81 ± 0.48	9	1.87 ± 0.54
03:00 h (T3)	5	1.51 ± 0.73	9	1.66 ± 0.54
06:00 h (T4)*	7	1.40 ± 0.40	8	1.71 ± 0.47
10:00 h (T6)*	8	1.75 ± 0.68	8	1.43 ± 0.23
14:00 h (T8)	5	1.42 ± 0.43	9	1.50 ± 0.61
16:00 h (T9)	6	1.64 ± 0.63	7	1.62 ± 0.80

Note: Data presented as mean ± standard deviation. *p<0.05, repeated measures ANOVA. Sample sizes (n) vary due to missing values at specific time points.

Table 2: Sleep characteristics of sleepy and not sleepy groups.

Variable	Sleepy (N=9)	Not Sleepy (N=9)	P-value
Men/Women	46176	46060	--
Sleep efficiency (%)	86.67 ± 6.3	78.1 ± 13.3	0.05
REM (%) in PSG	19.89 ± 3.44	13.8 ± 3.38	0.005
REM (%) 22:00-03:00 h	12.78 ± 6.1	10.4 ± 9.86	NS
REM (%) 03:00-06:00 h	29.33±6.48	16.3 ± 8.15	0.002
No. of REM episodes in MSLT	1 ± 1	0.22 ± 0.44	0.05
Total Sleep Time (min)	359.89 ± 39.7	307 ± 55.6	0.02
Sleep Latency (min)	19.56 ± 15.99	36.7 ± 24.87	NS
REM Latency (min)	101.89 ± 42.46	137.9 ± 75.76	NS
% Stage 2	58.67 ± 3.43	61.9 ± 4.65	NS
%SWS	20.56 ± 5.0	23.0 ± 3.01	NS
MSLT Sleep Latency (min)	5.44 ± 3.21	14.4 ± 2.51	<0.001*

Note: Data presented as mean ± SD. P-values from independent samples t-test. NS = Not Significant.
* Significant at p<0.001.

10:00 h, while the not sleepy group displayed the opposite pattern. Given that REM sleep typically occurs during the latter part of the night, it was decided to divide the PSG sleep test into two segments. This division aimed to compare REM sleep between the groups and investigate whether there is a relationship between REM sleep and the changing concentration of hypoxanthine.

Each overnight PSG recording was divided into two segments based on clock time: the first segment from lights-off (22:00 h) to 03:00 h and the second segment from 03:00 to lights-on (06:00 h). All participants had standardized lights-off at 22:00h and lights-on at 06:00h, resulting in consistent time-based segments across all subjects. In both segments, a higher percentage of REM was observed in the "sleepy" group compared to the Not sleepy group. Independent samples t-tests showed a significant difference in the percentage of REM stage in the second part of the sleep night among the sleepy subjects compared to the not sleepy group (p=0.002). In the MSLT, the sleepy group demonstrated significantly more REM sleep episodes compared to the "not sleepy" group (p=0.05). During the MSLT naps, independent samples t-tests revealed that the Sleepy group had significantly longer total sleep time compared to the "Not Sleepy" group (p=0.02).

Discussion

This study investigated the relationship between hypoxanthine levels and daytime sleepiness as measured by the Multiple Sleep Latency Test. We hypothesized that hypoxanthine, a non-peptide endozepine, would show differential concentrations between individuals with and without excessive daytime sleepiness. The main findings revealed that the "sleepy" group (MSLT latency ≤ 9 minutes) exhibited significantly lower hypoxanthine levels at 06:00h (end of nocturnal sleep) but elevated levels at 10:00h (late morning). Additionally, this group demonstrated higher REM sleep percentages during nocturnal PSG, more REM episodes during MSLT naps, and greater sleep efficiency. These findings suggest a potential association between hypoxanthine levels, REM sleep characteristics, and daytime sleepiness.

Our hypothesis was based on the established somnolent effects of benzodiazepines and the emerging evidence linking hypoxanthine to sleep homeostasis. Hypoxanthine, our endozepine of interest, acts as an endogenous ligand for GABAA receptors. Since GABAA receptor activation is a key mechanism underlying sedation and sleep promotion, we postulated that individuals experiencing excessive daytime sleepiness would exhibit altered hypoxanthine levels compared to non-sleepy individuals. Previous research demonstrating elevated hypoxanthine following sleep deprivation suggested its role as a marker of sleep pressure. We extended this concept to examine whether hypoxanthine levels would differentiate individuals with varying degrees of daytime sleepiness in the absence of acute sleep deprivation, thereby potentially serving as a biomarker for chronic sleepiness phenotypes.

The differential hypoxanthine concentration patterns observed between the sleepy and not sleepy groups reveal a complex regulatory mechanism that extends beyond simple elevation or reduction. The crossover pattern where the "sleepy" group exhibited significantly lower levels at 06:00h but higher levels at 10:00 h suggests dynamic, time-dependent differences in hypoxanthine metabolism between the groups rather than a static dysregulation. This inverse temporal relationship implies that the sleepy group may have a fundamentally different circadian or homeostatic regulation of hypoxanthine. The lower morning (06:00 h) concentrations in sleepy individuals, occurring at the end of nocturnal sleep when adenosine should be cleared and sleep pressure dissipated, paradoxically suggests either reduced hypoxanthine production, increased clearance, or altered metabolic pathways in this group. Conversely, the sharp rise by 10:00h in the sleepy group, diverging from the "not sleepy" group's pattern, may reflect compensatory mechanisms or differential adenosine catabolism dynamics. These concentration differences between groups independent of REM sleep considerations point to hypoxanthine as a potential independent biomarker of sleepiness that may capture aspects of sleep-wake regulation not fully explained by traditional polysomnographic measures. The consistency of group differences across multiple time points (22:00, 06:00, and 10:00h) strengthens the notion that hypoxanthine concentration patterns genuinely differentiate sleepy from non-sleepy phenotypes.

When examining the sleep structure in the sleepy and not sleepy groups, a significant difference is observed in a higher percentage of REM in the sleepy group in both PSG and MSLT, and a shorter REM latency in the Sleepy group was found (Table 2). It was found that the Sleepy group had a higher percentage of REM sleep in the second part of the night (03:00-06:00 h) compared to the "Not sleepy" group. Subsequently, a correlation between hypoxanthine levels and the percentage of REM sleep emerged, indicating that higher REM sleep percentages were associated with decreased hypoxanthine concentrations.

The temporal pattern of hypoxanthine in the sleepy group warrants particular attention. Hypoxanthine concentrations were notably depressed at 06:00 h (end of nocturnal sleep), then rose sharply by 10:00 h. This rise coincides with early morning hours when adenosine, a metabolic by-product, is typically cleared from the brain after extended sleep. Since hypoxanthine is a downstream metabolite of adenosine degradation, the morning increase may reflect adenosine catabolism during the adenosine-rich sleep period observed in this group. Alternatively, the elevated morning hypoxanthine could represent a compensatory mechanism following the REM-associated suppression of hypoxanthine observed during nocturnal sleep. The subsequent decline in hypoxanthine throughout the day may indicate ongoing homeostatic regulation of GABAergic tone. These temporal dynamics suggest that hypoxanthine may serve as a marker of circadian-homeostatic sleep-wake regulation [18-20].

REM sleep is a critical phase marked by muscle atonia, regulated by GABAergic neurons spread throughout key brain regions. These neurons are pivotal in suppressing muscle activity, ensuring the necessary muscular relaxation for physiological processes during this sleep stage. Moreover, GABAergic neurons modulate other neurotransmitter systems, such as the cholinergic system, contributing to the control of REM sleep patterns. This intricate neural communication is essential for promoting and sustaining REM sleep, facilitating smooth transitions between different sleep stages and wakefulness. However, long-term use of benzodiazepine medications, often prescribed for anxiety and insomnia, can hinder REM sleep by enhancing GABA's inhibitory effects in the brain. This suppression leads to reduced REM duration, prolonged Non-REM (NREM) stages, and a rebound effect of increased REM sleep upon withdrawal of the medication. Our findings demonstrate an inverse pattern: the "sleepy" group exhibited both reduced hypoxanthine concentrations and increased REM sleep percentages. This inverse relationship suggests that lower levels of hypoxanthine, as an endogenous GABAA receptor ligand, may result in decreased GABAergic inhibition, thereby permitting enhanced REM sleep expression. In other words, when hypoxanthine levels are low, there is less endogenous GABAergic tone to suppress REM sleep, analogous to the rebound increase in REM sleep observed when exogenous benzodiazepines are withdrawn [21-23].

In the realm of sleep disorders characterized by pronounced sleepiness, distinctions often hinge on REM sleep attributes. For example, narcolepsy typically manifests shortened REM latency, a heightened percentage of REM sleep, and higher episodes of REM in MSLT compared to sleepiness for example idiopathic hypersomnia due to other sleep disorders. These distinctive characteristics were evident in the sleepy group examined in the study, which displayed analogous REM sleep patterns [24].

Significantly, the study's findings identified a correlation between REM sleep and fluctuations in hypoxanthine levels, underscoring the relationship between sleepiness and hypoxanthine levels, particularly concerning REM sleep changes. Despite variations in hypoxanthine concentrations throughout the day, the research underscored a consistent link between sleepiness and hypoxanthine levels, notably influenced by REM sleep dynamics. As far as we know, this is the first time that endozepine has been described as a substance that may be a marker for sleepiness.

Given the limited existing research on hypoxanthine in the context of sleepiness, it remains plausible that further investigation could yield more conclusive evidence linking hypoxanthine to REM sleep and sleepiness. While orexin deficiency is well-recognized as a hallmark of type 1 narcolepsy [24], the notable decline in hypoxanthine levels following multiple REM episodes suggests a potential dual role for both orexin and hypoxanthine in understanding excessive sleepiness. Therefore, future studies should consider evaluating both markers concurrently to enhance our comprehensive understanding of the condition and its underlying mechanisms.

Moreover, establishing a connection between endozepines and excessive sleepiness could potentially lead to pharmacological advancements in future treatment modalities.

This exploratory study has limitations, including a small sample size (n=18) which may limit generalizability. However, the controlled laboratory conditions and repeated measures design provide reliable insights into hypoxanthine dynamics. Larger prospective studies will be needed to confirm these findings and establish clinical utility.

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