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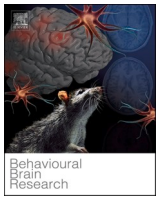

Circadian variations influence anxiety-related behaviour, olfaction, and hedonic response in male Sprague–Dawley rats

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ABSTRACT

Introduction: Despite the acknowledged impact of circadian rhythms on various aspects of life, behavioural tests with laboratory animals often overlook alignment with their natural activity patterns. This study aims to evaluate the influence of circadian variations on the results, validity, and reliability of different behavioural tests in rats.
Methods: Three behavioural tests, the Light-Dark Box Test (LDB), assessing anxiety-related behaviour and locomotor activity; the Buried Pellet Test (BPT), revealing olfactory abilities and motivation issues; and the Sucrose Preference Test (SPT), studying the anhedonic response, were employed to encompass multiple daytime-dependent behavioural aspects in male Sprague-Dawley rats.
Results: Our findings underscore distinct circadian effects on locomotor activity, exploratory behaviour, olfactory acuity, motivation, and hedonic response. Notably, anxious behaviour remained unaffected by daytime conditions. Furthermore, decreased data variance was found to be correlated with conducting behavioural tests during the subjects' active phase.
Discussion: This study demonstrates extensive circadian influences on nearly all parameters investigated, coupled with a significant reduction in data variability during the active phase. Emphasising the importance of aligning experimental timing with rats' natural activity patterns, our results suggest that conducting tests during the active phase of the animals not only refines test sensitivity ~~, reduces stress,~~ and provides more representative data, but also contributes to ethical animal research (3 R) and improves test relevance. This, in turn, enhances the reliability and validity of experimental outcomes in behavioural research and promotes animal welfare.

1. Introduction

Rats are an indispensable tool in contemporary scientific research, ranking among the best-studied animals. To date, PubMed lists an extensive repository of over 1.8 million publications incorporating rats of any kind, with approximately 15 % of these studies dealing with behavioural aspects (PubMed, November 2nd, 2023). Many aspects potentially affecting rat behaviour are well studied, like influences of sex, age, or strain [1–4]. However, there is a notable gap in scientific inquiry regarding the potential impact of circadian rhythm on the outcomes of behavioural tests conducted in rats. Indeed, a systematic literature review revealed only 73 studies addressing the influence of circadian variations on the results of behavioural tests in rats published since 1975. Within these studies, only three systematically compared behavioural results obtained during the active phase of the animals with those acquired during their inactive phase [5–7]. In addition, in a recent review, the authors stated that the time-of day reporting rate in animal studies is still as low as ~ 6 % [8].

While in mice, the influence of circadian variations on various behavioural domains was extensively studied, less effort has been made in rats [9–11]. The present study aims to systematically investigate the impact of circadian variation on the outcomes of different behavioural tests in rats, allowing researchers to adjust their specific behavioural tests to the needs and abilities of their research subjects and their research questions. In contrast to previous research in the field, this study exclusively focuses on circadian variations, excluding additional covariates such as medication or genetic models. Moreover, we performed a set of three behavioural tests to provide a comprehensive overview of several behavioural domains in male Sprague-Dawley rats.

We hypothesise that conducting behavioural tests during the active

Abbreviations: BPT, Buried Pellet Test; LDB, Light-Dark Box; SFPT, Surface Pellet Test; SPT, Sucrose Preference Test.

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phase of the animals enhances test sensitivity due to increased motivation, cooperation, and commitment among the animals and also affects test outcomes in general. Furthermore, active-phase-testing might allow researchers to observe behaviours that are more representative of the animal's natural behaviour, as they are more likely to engage in activities when tested in their active phase [12]. Additionally, animal welfare can be increased by sticking to their natural rhythm since testing rats during their inactive period can induce stress by disrupting their natural circadian rhythm, impacting their physical and psychological well-being [12,13]. Conducting tests during their active phase helps to minimise stress and provides a more comfortable testing environment. By acknowledging and addressing the influence of circadian variations while planning the methodology of behavioural tests, researchers can refine their experimental approaches, ultimately advancing tests' sensitivity, reliability, reproducibility, and overall quality.

2. Materials and methods

2.1. Systematic literature review

All literature searches were conducted in PubMed on November 2nd, 2023 (https://pubmed.ncbi.nlm.nih.gov/; see Fig. 1 for review synopsis). To initially estimate how many publications paid attention to the diurnal timing of experiments, a PubMed search limited to the year 2022 as a representative period (at time of literature review most recent year with completed study publication) and to the species rat as an exemplary laboratory animal was conducted utilising the following search terms: *(behavioural test) AND (rat) AND (2022) NOT (review),* resulting in 686 publications in total. 109 papers were excluded because publications either were reviews or retracted, did not include rats or behaviour tests with rats, were not available (in English), had study designs with other than 12-hour light-dark rhythms, or repeated testing. A second search strategy was applied to obtain a more detailed picture: To determine the number of publications evaluating the effects of circadian variations on the outcome of behavioural tests in rats, the following search term was utilised: *((rat) AND (behavioural testing)) AND (circadian variation)*. All publications (73 in total) from the beginning of records were considered and systematically considered for study design, species, type of behavioural test(s), and their relevance to the research question. To verify our methodology, we performed a second additional systematic literature research on the 11th of April 2024, utilising the broader search term *(rat) AND (behaviour) AND (circadian) AND ("night phase" OR "dark phase" OR "at night" OR "diurnal variation" OR "circadian phase" OR "time of day")*, resulting in 1030 offers. Due to the increasing unreliability of the PubMed search algorithm, we only analysed the first 300 publications.

2.2. Animals

All rats were kept in a controlled environment with temperature and humidity regulation, following a 12-hour light-dark cycle (lights on 6 a.m. with 250 lx and lights off 6 p.m.), and had *ad libitum* access to food and water. All animal experiments adhered to the European Directive (2010/63/EU) and ARRIVE guidelines and were approved by the local animal care committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern LALLF, Germany: M-V/7221.3–1–002/21). For all experiments wild-type male Sprague-Dawley rats aged three months (310–490 g) bred at the central animal facility of the University Medical Center Rostock were utilised. A total of 15 rats went into final analyses. All animals were group-housed.

2.3. Behavioural tests

The ACTIVE cohort (n=9) was constantly handled and tested in the first hours of their active phase under quiet and dark conditions. To ensure necessary behavioural observations, all work was done under red lights. In contrast, the INACTIVE cohort (n=6) underwent all handling

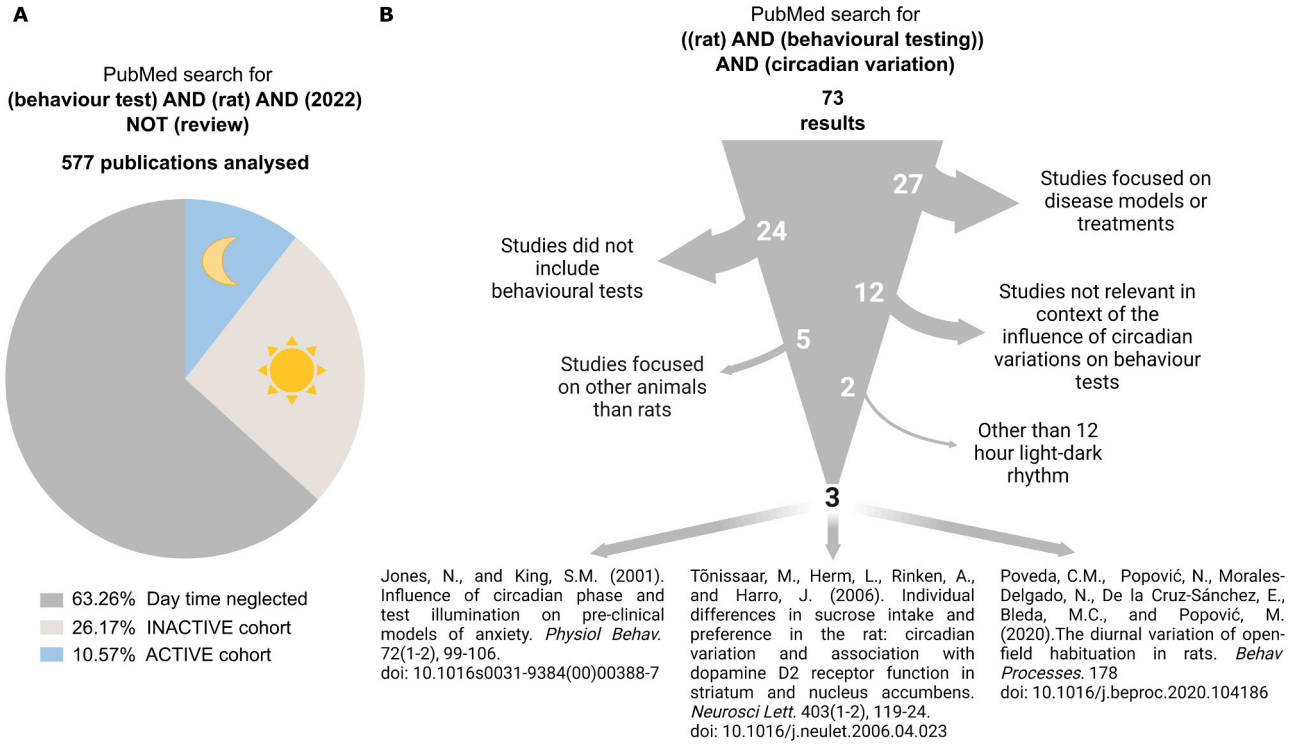

Fig. 1. Representation of literature search. (A) Incorporation of daytime considerations in rat behaviour tests within the landscape of studies published in 2022. Studies either neglected the daytime of behaviour tests (grey) and consequently did not mention a specific activity phase or clearly state whether they conducted their behavioural tests during the INACTIVE (light grey) or the ACTIVE phase (blue) of the subjects. (B) Literature review derived from PubMed repository, encompassing scientific publications on circadian variations in rats from 1975 onward (created with biorender.com).

and behavioural tests during the first hours of their inactive phase in a quiet and lit room with the standard light conditions during daytime (250 lx). Prior to the first behavioural test, rats were allowed a one-week acclimatisation period to recover from potential shipping-related stress and to adjust to their new surroundings. The ACTIVE group was moved to the testing room at the beginning of the dark phase (6 p.m.), whereas the INACTIVE group was relocated to the testing room at 6 a.m. Hence, the adjustment period of one hour before the experiment occurred during the same time of day as the subsequent behavioural assessment. Three behavioural tests were chosen to cover as many behavioural domains as possible. In between trials, rats were allowed to recover for two days. The experimental timeline for both cohorts is schematically depicted in Fig. 2.

2.3.1. Light-dark box test (LDB)

The LDB is a behavioural test commonly used to assess both anxiety-related behaviour and exploratory behaviour in rodents [14]. Rats were transferred to the experiment room for one hour before testing. The self-constructed acrylic Light-Dark Box apparatus consisted of two compartments, with two-thirds (34 cm×51 cm) of the total area exposed to 65 lx illumination. In contrast, the remaining one-third (17 cm×51 cm) remained in darkness with 0 lx [15]. A non-transparent wall with a door measuring 8 cm×8 cm separated both compartments. To minimise any neophobic reaction to the test environment, the box was previously marked by male rats that were not part of the actual experiment. Additionally, the apparatus was only wiped with wet towels between trials [16]. Rats were placed individually in the centre of the illuminated area, facing away from the opening. A 10-minute test session was conducted, during which the following parameters were recorded: latency to first entrance into the dark compartment, total time spent in any of the compartments, number of compartment switches, number of rearings, and total distance travelled. All trials were captured and subsequently analysed with EthoVision XT 8.5 software (Noldus, Wageningen, Netherlands).

2.3.2. Buried pellet test (BPT) and surface pellet test (SFPT)

The BPT is designed to measure olfaction by testing the animal's ability to locate a buried food pellet using olfactory cues [17]. Two days before testing, rats received three daily Froot Loops (Kellogg's®) feedings to acquaint them with the preferred food's scent and taste and avoid potential neophobia during the test. Animals were set on food restriction ten hours before testing [18]. One hour before the trial, rats were housed in the experiment room in their home cages. A new cage (type III) with 6 cm thick, fresh bedding was prepared for each subject. Just before testing, a single Froot Loop was concealed approximately 4 cm below the surface of the bedding. Rats were placed on the opposite side of the cage. Time was measured until the rat found and retrieved the pellet. After testing all rats, a similar procedure was performed for the SFPT. However, the pellet was not buried but placed openly on the surface of the bedding. New positions for the rat and pellet were selected, remaining again constant for all subjects [19].

2.3.3. Sucrose preference test (SPT)

The SPT is often employed as a measure of depressive-like behaviour or as an indicator of an animal's response to stress [20]. From two days before the trial until the final day of testing, all rats were constantly provided with two bottles of plain water to familiarise them with the opportunity to choose between two bottles [21]. One hour prior to the test, rats were separated and placed in new cages. Once again, two bottles of plain water were made available. Directly before the test, fresh bottles were filled with either plain water or a 2 % sucrose solution, weighted, and placed side by side on the cages. After the test session, the bottles were weighed again and thoroughly cleaned [22]. The test was conducted over four consecutive days, with each session lasting four hours [23]. The side-by-side location of the bottles was switched daily to avoid any potential bias, e.g., due to side preferences. The mean of all four test days was calculated for the following parameters: consumption of plain water or sucrose water and body weight.

2.4. Statistical analysis

Data was analysed and visualised with GraphPad Prism (GraphPad Software Inc.) and Inkscape 1.3. Grubb's Test with a 1 % quantile was employed to test for potential outliers. To assess the effect of daytime on test results, unpaired two-tailed Student's t-tests or Welch's test were performed, as appropriate. The values of the coefficient of variation were compared with a paired Student's t-test. $P<0.05$ was deemed statistically significant. All data is expressed in boxplots with a central mark at the median, bottom, and top edges of the boxes at 25th and 75th percentiles, respectively, and whiskers at the minimum/maximum. Each dot represents an individual value. Outliers are marked as white dots. A detailed description of the statistics can be found in the Supplementary Material (Table S1).

3. Results

3.1. Systematic literature review on circadian variations on rat behavioural testing

To obtain an overall impression on the percentage of publications paying attention to the diurnal timing of behavioural experiments, a systematic literature search limited to the year 2022 as an exemplary time period and to rats as one major laboratory animal was conducted: PubMed lists 686 publications on rat behavioural tests for the year 2022. Only around 30 % of them specified the activity time of the animals during which they conducted their behavioural tests, and merely 10 % of researchers performed their tests during the animals' active phase (Fig. 1**a**). A further, more detailed literature review on the influence of

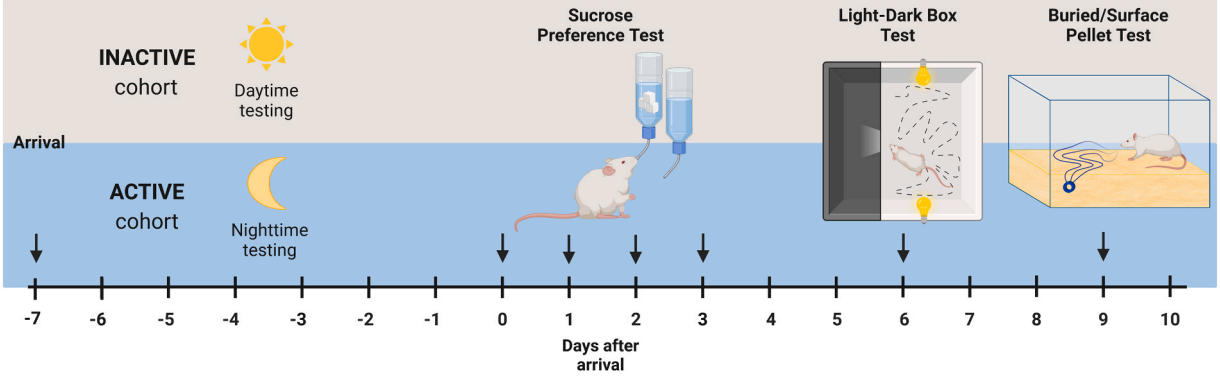

Fig. 2. Experimental design of the study (created with biorender.com).

circadian variations on the outcomes of behavioural tests detected only 73 studies addressing this question since 1975 (Fig. 1**b**). Only three out of these 73 reports systematically compared behavioural results obtained during the active phase of the animals with those acquired during their inactive phase. All three presented studies focused on either one behavioural aspect or one specific behavioural test [5–7]. In the second literature research, the same results were obtained. No additional papers were found within the initial 300 publications describing circadian variations on results of behavioural tests conducted with rats (Suppl. Fig. S1).

3.2. *Effects of circadian variations on anxiety and locomotor activity*

From the LDB, multiple parameters were analysed to evaluate both anxiety-related behaviour and locomotor activity. Time spent in the illuminated compartment served as an indicator for anxiety-related behaviour. The INACTIVE cohort exhibited no alterations in anxiety-related behaviour levels compared to the ACTIVE cohort (INACTIVE: 246.70±98.02 s, ACTIVE: 283.00±78.15 s, P=0.4542, Fig. 3**a**). Additionally, the latency to the initial compartment switch, sometimes also employed as a measure of anxiety-related behaviour, was not influenced by daytime (INACTIVE: 29.67±22.78 s, ACTIVE: 29.63±33.18 s, P=0.6354, **Suppl.** Fig. 2**b**).

However, regarding locomotor activity, rats displayed increased numbers of compartment switches (INACTIVE: 14.67±5.82, ACTIVE: 24.25±7.05, P=0.0192) and rearings (INACTIVE: 30.17±6.68, ACTIVE: 48.25±10.15, P=0.0026) during the ACTIVE period. Furthermore, the total distance travelled was significantly elevated during the subjects' ACTIVE phase (INACTIVE: 3.00±0.59 m, ACTIVE: 6.12±0.4671 m, P<0.0001; representative trails are depicted in Fig. S2a).

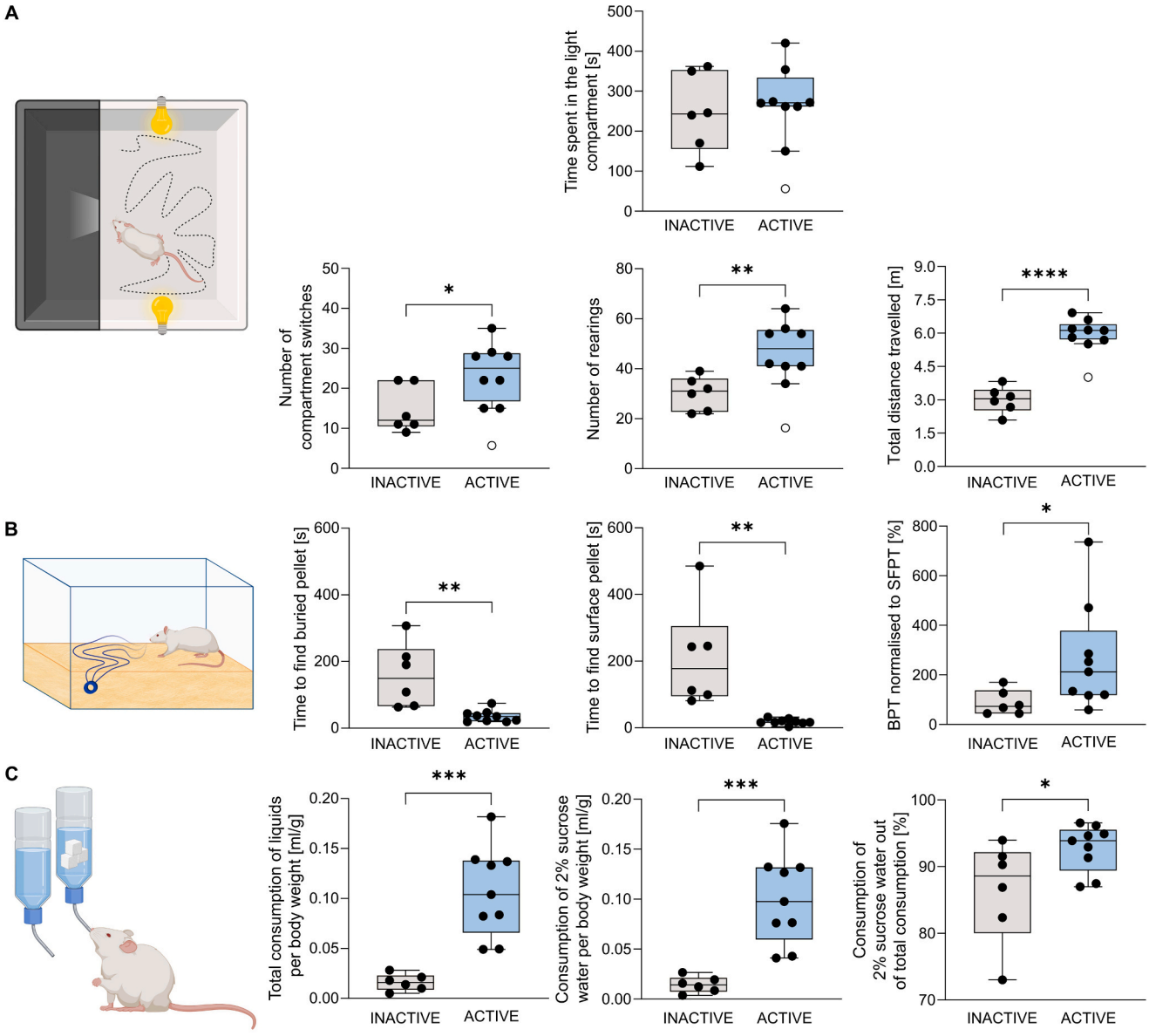

Fig. 3. Behavioural parameters obtained in (A) the Light-Dark Box Test, (B) the Buried/Surface Pellet Test (BPT/SFPT), and (C) the Sucrose Preference Test, including the schematic test setup of each behavioural test. (A) Time a rat spent in the light compartment, compartment switches, number to of rearings, and total distance a rat traveled in the Light-Dark Box Test. (B) Time the rat needed to retrieve the buried pellet in the BPT, time the rat needed to find the surface pellet in the SFPT and time to find the buried pellet normalized to time to find the surface pellet. (C) Total liquid consumption normalized to body weight, consumption of 2 % sucrose water normalized to body weight, and percentage of consumed 2 % sucrose water. Data are presented as median and interquartile ranges with all individual values; outliers were detected with Grubbs Test (1 % quantile) and are indicated as white dots. For the Sucrose Preference Test, a mean of all four days of the trial is shown. Cohorts were compared with unpaired Student's t-test or Welch's t-test, as appropriate (created with biorender.com).

3.3. Effects of circadian variations on olfaction and motivation

The BPT serves as a tool for evaluating olfactory acuity. In this diurnal assessment, the INACTIVE cohort displayed a prolonged duration in locating the hidden/buried pellet compared to their ACTIVE counterparts (INACTIVE: 158.20±96.06 s, ACTIVE: 35.77±17.80 s, P=0.0273, Fig. 3b). Similar findings were obtained in the SFPT (time to retrieve the displayed pellet; INACTIVE: 210.80±152.60 s ACTIVE: 18.40±8.29 s, P=0.0273). To determine potential hyposmia, the BPT latency was normalised to the SFPT latency. With this approach, olfaction can be quantified independently of other parameters, possibly affecting retrieval time, such as motor impairment or differences in motivation. Here, we found a significant difference (INACTIVE: 88.27 ±50.01 %, ACTIVE: 264.40±214.10 %, P=0.0409).

3.4. Effects of circadian variations on liquid consumption and anhedonia

The SPT evaluates the consumption of sweetened liquid compared to plain water as a measure of (an-)hedonia. The absolute quantity of ingested liquids normalised to individual body weight exhibited a significant increase during the ACTIVE phase of the animals (INACTIVE: 0.01606±0.00822 ml/g, ACTIVE: 0.1065±0.04473 ml/g, P=0.0017, Fig. 3**c**). Furthermore, both absolute and relative sucrose consumption were significantly increased in the ACTIVE compared to the INACTIVE cohort (INACTIVE: 0.01444± 0.00811 ml, ACTIVE: 0.10000 ±0.04513 ml, P=0.0004; INACTIVE: 86.35±7.67 %, ACTIVE: 92.76 ±3.52 %, P=0.045). In contrast, body weight did not differ between the cohorts (INACTIVE: 458.8±14.90 g, ACTIVE: 418.9±59.05 g, P=0.133, Fig. S2c).

3.5. Effects of circadian variations on accuracy of behavioural tests

The coefficient of variation is a measure of data variability. Here, we compared the variation within each parameter from the above-mentioned behavioural tests from the ACTIVE and INACTIVE testing (INACTIVE: 41.18±21.07 %, ACTIVE: 30.12±16.82 %, P=0.0016, Fig. 4**)**. Data obtained during the ACTIVE phase of the animals were approximately 11 % less scattered than data gained during the INACTIVE phase.

4. Discussion

Behavioural studies with rats and rodents, in general, are valuable tools in modern biomedical research. Although commonly practised, the methodology, particularly with regard to the timing of assessments during the day in consideration of the circadian rhythm of the laboratory animal model, varies across the literature. These variations, however, pose challenges when comparing studies, as they may impact the reliability and validity of the findings, potentially introducing confounding factors and hindering the ability to transfer the results to one's own research. It is noteworthy that our systematic literature review for the representative period of the year 2022 reveals that only approximately 30 % of the reports specified the activity time of the animals during which the behavioural tests were conducted, and merely 10 % of studies performed the tests during the animals' active phase. Moreover, our literature search clearly demonstrated a large research gap on the influence of circadian variations on the outcome of behavioural tests in rodent laboratory animals: we detected only three systematic studies published since 1975, which compared behavioural results in rats obtained during the active with those obtained during the inactive phase of the day [5–7]. These reports limit their studies to either one behavioural aspect or one specific behavioural test.

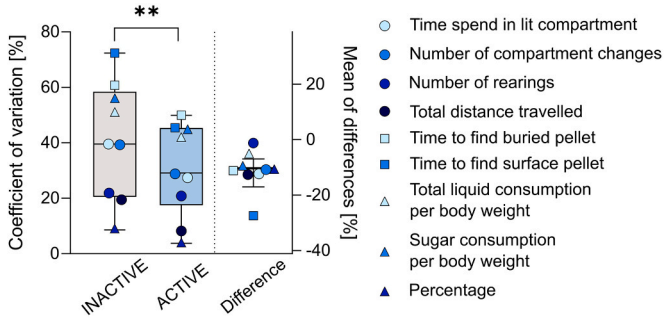

Fig. 4. Coefficient of variation (CV) of parameters from the Light-Dark Box Test (circles), the Buried Pellet Test (squares), and the Sucrose Preference Test (triangles) in the two cohorts (INACTIVE and ACTIVE) compared with a paired students t-test (left side), and differences of paired CVs as percentage (right side).

Significant circadian behaviour research in mice has shown diurnal variations in behaviours such as hedonic drinking and anxiety-related responses. For example, mice exhibit increased sucrose preference during their active phase and display fewer anxiety-related behaviours in the light-dark box test when tested at night [10,11]. Furthermore, diurnal variations in pain perception as a behaviour domain were extensively reported [24,25]. These findings underscore the importance of considering circadian influences in rodent behavioural studies. In our study, we therefore examined the influence of circadian variations on the results, validity, and reliability of behavioural tests using rats as one major laboratory animal species. To this end, we conducted various behavioural tests to cover as many behavioural domains as possible.

Across all tested behavioural domains, we only found one parameter (anxiety-related behaviour) unaffected by diurnal variations. All other parameters displayed an association with diurnal test execution. These effects can be succinctly summarised by three fundamental observations: during inactive periods, rats manifested a prominent reduction in overall locomotor activity, drinking, and feeding behaviours. Consequently, parameters such as locomotor activity and exploratory behaviour exhibited pronounced variations depending on the time of testing, as presented in the LDB. Alternated feeding and drinking behaviours during their less active phases impact the results of behaviour tests relying on those stimuli, such as the BPT/SFPT and the SPT. We hypothesise that these variations can be mutually attributed to reduced motivation during the subjects' inactive period, as it has been previously described in mice [26,27]. Considering motivation in interpreting results becomes even more evident when analysing the results of the BPT/SFPT. Excluding alternations in food motivation by normalising the BPT to the SFPT indicates that prolonged pellet-finding times in the INACTIVE cohort are not linked to olfactory impairment [28]. Normalising of time reveals unexpectedly increased olfactory sensitivity during the initial hours of the inactive phase, similar to what has previously been reported by Francois *et al.* [29].

Another argument for behavioural testing during the subjects' active phase is animal welfare: aside from an overall reduced total liquid consumption in the inactive phase, the SPT strongly suggests a more pronounced anhedonic response, represented by the percentage of consumed sucrose water. In the literature, it has been described that being less responsive to reward is a symptom of stress (18, 19, 26). Therefore, testing during their active phase aligns with promoting animal welfare, as it reduces stressors associated with testing conditions by respecting intrinsic behavioural patterns.

Testing during the active phase of animals not only influences the overall outcomes of behaviour tests, as shown in the BPT and SPT but also reveals a significant difference in the coefficients of variation between the ACTIVE and INACTIVE cohorts. The reduced dispersion in data during the active phase implies a higher level of precision in test results, enhancing the reliability and interpretability of experimental outcomes. Consequently, behavioural tests conducted during the active phase yield more accurate and reproducible results. This offers a potential reduction in the number of animals required for experimentation and aligns with the ethical principle of the 3 Rs (Replacement,

Reduction, Refinement) in animal research [30].

While our study provides valuable insights into the influence of circadian variation on behavioural tests in rats, it is essential to acknowledge its limitations. Our use of only male rats of a single strain and age group may limit the generalizability of our findings, as there could be sex- and age-specific responses to circadian variations in behaviour (28, 29). Furthermore, the small number of animals and behavioural tests in our study could affect the statistical power and robustness of the results.

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CRediT authorship contribution statement

Mareike Fauser: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Alexander Storch:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition. **Franz Markert:** Writing – review & editing. **Meike Statz:** Writing – review & editing, Validation, Investigation. **Hanna Weber:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Paperpal in order to improve the language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2024.115134.

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