Pharmacology, Biochemistry and Behavior 178 (2019) 19–29

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Effects of light and dark phase testing on the investigation of behavioural paradigms in mice: Relevance for behavioural neuroscience

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ARTICLE INFO

Keywords:
Behavioural neuroscience
Daily rhythm
Testing time
cFos

ABSTRACT

Different timing and light phases are critical factors in behavioural neuroscience, which can greatly affect the experimental outcomes of the performed tests. Despite the fact that time of testing is one of the most common factors that varies across behavioural laboratories, knowledge about the consequences of testing time on behavioural readouts is limited. Thus, in this study we systematically assessed the effect of this factor on the readout of a variety of elementary and recurrent behavioural paradigms in C57Bl/6 mice. Furthermore, we investigated potential neuronal correlates of this phenomenon by analysing how testing time influences the expression pattern of genes relevant for neuronal activation functions and the control of brain circadian rhythms. We show that animals tested in the light phase display reduced social approach behaviour and sensorimotor gating and increased locomotor activity, whereas anxiety-related behaviour and working memory are not affected. In addition, animals tested in the light phase also exhibit increased locomotor response to systemic amphetamine treatment, which is paralleled by alterations in the expression patterns of tyrosine hydroxylase (TH) and dopamine transporter (DAT) in the Nucleus Accumbens (NAc) and/or Midbrain (Mid). Lastly, we observed that neuronal activation, indexed by the gene expression levels of cFos, was increased in the NAc and Mid of animals tested during the light phase. Our data thus suggest that daily alterations in gene expression in mesolimbic brain structures might be involved in the different behavioural responses of mice tested in the light- versus the dark-phase. At the same time, our study adds further weight to the notion that the specific timing of testing can indeed strongly affect the readout of a given test. As comparison and reproducibility of findings is pivotal in science, experimental protocols should be clarified in detail to allow appropriate data comparison across different laboratories.

1. Introduction

Behavioural neuroscience implements a variety of cross species translational paradigms that have been developed for the identification and characterization of neuropsychological, psychopharmacological and cognitive functions implicated in normal behaviour and psychiatric disorders. The most common species used in laboratory behavioural testing are rodents, mainly inbred mice and rat strains. Most rodents are nocturnal animals, meaning that they spend most of the time during the light-phase of the day sleeping, whereas during the dark-phase they are active and carry out the necessary tasks essential for surviving. Indeed, like all mammals, rodents experience daily rhythmic changes in sleep-wake cycles, hormonal pulses, nervous activity and body temperature under the control of the circadian clock. This circadian pattern is also evident in common laboratory rodents (Kopp et al., 1998). Among various physiological events, circadian clocks also align behavioural processes with the day/night cycle, resulting in a characteristic oscillatory trend in behavioural performance (Bass and Takahashi, 2010; Reppert and Weaver, 2002). Against this background, it is not surprising that circadian timing and daily light phases are critical factors in behavioural neuroscience, which can, in turn, greatly affect the experimental outcomes of the performed tests. This statement acquires even greater importance when considering that one of the most common factors that varies across behavioural studies is the time of day (e.g. phase of the light/dark cycle) during which the animals are tested. Importantly, however, a limited amount of studies have addressed this issue (Ago et al., 2014; Brooks and Dunnett, 2009; Ota et al., 2015; Valentinuzzi et al., 2000; Valentinuzzi et al., 2004; Bilu and Kronfeld-Schor, 2013), and often yield conflicting results (Arakawa et al., 2007; Chabot and Taylor, 1992; Frankland and Ralph, 1995; Yang et al.,

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https://doi.org/10.1016/j.pbb.2018.05.011
Received 24 January 2018; Received in revised form 7 May 2018; Accepted 18 May 2018
Available online 18 May 2018
0091-3057/

2008; Adams et al., 2008; Weiss et al., 1999). Interestingly, a recent study highlights that environmental enrichment affects exploratory and emotional behaviours, such effects, however, are only evident when mice are tested during the light phase (Loss et al., 2015). Similarly, a recent study found opposite effects of restraint stress on anxiety-like behaviours depending on the light cycle (Ota et al., 2015). This suggests that not only the behavioural testing outcomes might be different depending on the phase of the day in which they are conducted, but that the experimental manipulations might even interact with the different daylight phases in determining different behavioural outcomes (Loss et al., 2015). These findings thus highlight the potential impact that different testing regimes might have on behavioural phenotypes and underline how this methodological and potentially confounding factor may affect reproducibility of results across different laboratories.

On this basis, we tested one of the most widely used mouse strains, namely C57BL/6 mice, in a variety of elementary and recurrent behavioural paradigms with the aim to assess whether the specific timing of the behavioural testing actually affects the readout of a given test. Thus, two separate cohorts of animals were tested at different daylight phases (i.e. dark versus light phase). Due to limitations arising from animal welfare regulations, the current study does not include the assessment of depression-like behaviour, but focuses on the assessment of the following parameters: locomotor activity in the open-field, anxiety in the light/dark box test, spatial working memory in the Y-maze, social interaction and sensorimotor gating in the form of a prepulse inhibition test. These specific behavioural tasks were chosen based on their relevance for the investigation of translational behavioural phenotypes in preclinical research and the long-standing experience of our laboratory in modeling behavioural clusters relevant to neurodevelopmental disorders (Richetto et al., 2013; Richetto et al., 2015; Richetto et al., 2017; Stadlbauer et al., 2014; Weber-Stadlbauer et al., 2017). Of note, the current study implements the most common parameters of behavioural testing used across different laboratories. For example, dark phase testing was conducted under dimly lit (and sometimes brightly lit) circumstances, which responds to the necessity of using light as an aversive stimulus in anxiety related tests, and the scarce use of infrared detection systems in the majority of laboratories. While this choice increases the relevance of the current findings, it does add a level of complexity due, for example, to the effect of light exposure in the dark phase on the entrainment of the animals to the light-dark cycle. These possible confounders, however, do not affect the main message of the study, and are adequately addressed in the discussion.

Secondly, our study aimed at investigating potential neuronal correlates of this phenomenon by analysing how specific testing time is paralleled by the expression pattern of certain genes relevant for (i) neuronal activation functions, and (ii) the control of brain circadian rhythms. While a central role of these systems in putative daily behavioural performance fluctuation is only starting to be investigated (Mulder et al., 2013), we decided to focus on these candidates because of different scientific evidence suggesting that they could be involved in such phenomenon: firstly, the immediate early genes *Arc* and *cFos* are rapidly and transiently evoked by a wide range of external stimuli activating a synapse (Perez-Cadahia et al., 2011). Both genes are pivotal for neuronal activation and are implicated in bridging the gap between early and late responses in neuronal plasticity events (Robertson, 1992; Kaczmarek, 1993) that underlie complex behavioural processes such as learning and memory. Against this background, different levels of expression of these genes during night and day following behavioural testing could result in – or be a marker of – varying degrees of neuronal activation in response to different stimuli, thus participating in the oscillatory nature of behavioural performance between different daylight phases. Secondly, we investigated the gene expression levels of core circadian clock genes, namely Clock, Bmal1, Per1 and Per2. Specifically, Clock and Bmal1 form a transcriptional activator complex that positively regulates a wide variety of targets that are the output of the circadian clock itself (Takahashi, 2015). Among these, the transcriptional activator complex promotes the transcription of Per1 and Per2, which, in turn, exert a negative feedback-loop, together with the Crypthochrome proteins, on the transcription of Clock and Bmal1, perpetuating the clock cycle (Reppert and Weaver, 2002). The expression profile of these circadian players has been more extensively studied in hypothalamic areas, including the suprachiasmatic nucleus (considered the master regulator of the circadian clock), brainstem regions and cerebellum (Mure et al., 2018; Zhang et al., 2014), while reports on their oscillatory pattern in brain areas crucial for behavioural functions are still limited (Li et al., 2013).

Lastly, prompted by our results, two additional cohorts of animals were tested at different daylight phases (i.e. dark versus light phase) in the amphetamine sensitivity test, and the expression pattern of two main players of the dopaminergic system was investigated. In detail, we focused on the rate-limiting enzyme of the dopamine synthesis pathway, tyrosine hydroxylase (TH), and the dopamine transporter (DAT), which have also been shown to be regulated by the circadian clock (McGeer and McGeer, 1966; Sidor et al., 2015; Sleipness et al., 2007; Landgraf et al., 2016a). In turn, daily rhythms in dopamine production and release could provide an important daily drive influencing a wide array of physiological functions, including emotional, attentional, cognitive and reward-related behaviours (Nieoullon, 2002; Spanagel and Weiss, 1999; Landgraf et al., 2016b).

In conclusion, our study provides a comprehensive phenotyping strategy to test the behavioural and molecular consequences of light- and dark phase testing in male and female C57Bl6 mice. Our results elucidate potential differences in behavioural read-outs carried out in light- versus dark phase that are crucial to enable comparability and reproducibility of data across research facilities.

2. Materials and methods

2.1. Animals

C57BL/6N mice were used throughout the study. C57BL/6N males and females were originally obtained from Charles River Laboratories (Germany) and maintained in the animal facility for a period of at least two weeks for acclimatization before any behavioural experiments were performed. All animals were held in IVC (Individually Ventilated Cages) in groups (3–4 per cage) and in a temperature- and humidity-controlled (21 ± 1 °C; 55 ± 5%) vivarium. Food and water were always accessible ad libitum throughout all experiments. All the animals were maintained under a reversed light-dark cycle (lights off: 09:00 am to 09:00 pm). After the acclimatization period, animals were randomly assigned to the two experimental groups and underwent behavioural testing at opposite daylight phases: one group was tested in the dark phase (experiments started at 11.00 am) and one group in the light phase (experiments started at 11.00 pm). Two additional groups of animals (one tested during the dark phase and one during the light phase) were subjected to the AMPH sensitivity test or the Open Field test. Both cohorts were then used for post-mortem gene expression analyses. All procedures were approved by the Cantonal Veterinary Office of Zurich and are in agreement with the principles of laboratory animal care in the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 86-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

2.2. Behavioural testing

Behavioural tests were performed in the order presented below, with a minimum resting time of four days between the tests. Behavioural tests did not commence in the first 2 h before and after the switch of light-phases.

2.2.1. Light-dark Box

The light-dark box (LDB) test is one of the most widely used tests to measure innate anxiety-like behaviour in rodents. The LDB test is based on the natural aversion of mice to brightly illuminated areas and on their spontaneous exploratory behaviour in novel environments. The LDB consisted of four identical two-way shuttle boxes (30 × 30 × 24 cm; Multi Conditioning System, TSE Systems GmbH, Bad-Homburg, Germany). The boxes were separated by a dark plexiglass wall, and interconnected by an opening (3.5 × 10 cm) in the partition wall, thus allowing the animal to freely traverse from one compartment to the other. This wall divided the compartment into a dark (1 lx) and a brightly illuminated (100 lx) compartment. Each mouse was placed in the center of the dark compartment and was allowed to move freely for 10 min. Innate anxiety was indexed and calculated based on the percent time spent in the light compartment during the 10 min of exploration ([time spent in light compartment/total time] × 100) (Takao and Miyakawa, 2006). Total distance moved was measured to ascertain that percent time spent in the light compartment was not confounded by changes in locomotor activity.

2.2.2. Open field

The open field exploration test is a widely used behavioural assay to evaluate locomotor responses to novel environments in rodents (Belzung and Griebel, 2001). The test was conducted in 4 identical square arenas (40 × 40 cm) surrounded by walls (35 cm high). The apparatus was made of white Plexiglas and was located in a testing room under lighting (28 lx in the center zone, 20 lx in the corner of the maze). A digital camera was mounted directly above the four arenas. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus Information Technology, The Netherlands) tracking system. The animals were gently placed in the center of the arena and allowed to freely explore for the duration of 60 min. The dependent measure was the mice's total distance moved in the entire arena.

2.2.3. Spatial working memory in the Y-maze

Short-term spatial recognition memory was assessed using a Y-maze test as established and validated before (Richetto et al., 2013; Richetto et al., 2015). This test uses the natural tendency of rodents to explore novel over familiar spatial environments. The apparatus was made of transparent Plexiglas and consisted of three identical arms (50 × 9 cm; length × width) surrounded by 10-cm high transparent Plexiglas walls. The three arms radiated from a central triangle (8 cm on each side) and were spaced 120° from each other. A removable opaque barrier wall was used to block access to each arm from the central area. The maze was elevated 90 cm above the floor and was positioned in a dimly-lit room (16 lx in the maze) enriched with distal spatial cues. A digital camera was mounted above the Y-maze apparatus. Images were captured at a rate of 5 Hz and transmitted to a PC running the EthoVision tracking system (Noldus Information Technology, The Netherlands), which calculated the time spent and distance moved in the arms and center zone of the Y-maze.

The short-term spatial recognition memory test in the Y-maze consisted of two phases, called the sample and choice phases. The allocation of arms (start, familiar, and novel arm) to a specific spatial location was counterbalanced across the experimental conditions.

Sample phase: The animals were allowed to explore two arms (referred to as “start arm” and “familiar arm”). Access to the remaining arm (“novel arm”) was blocked by an opaque barrier wall. To begin a trial, the animal was introduced at the end of the start arm and was allowed to freely explore both the start and the familiar arms for 5 min. The animal was then removed and kept in a holding cage prior to commencement of the choice phase. The barrier door was removed and the maze was cleaned to avoid olfactory cues.

Choice phase: The animal was introduced to the maze following a retention interval of 1 min. During the choice phase, the barrier wall was removed so that the animals could freely explore all three arms of the maze for 5 min. The animal was then removed from the maze and returned to the home cage. The maze was cleaned in preparation for the next trial.

On each trial, the time spent in each of the three arms was recorded. The relative time spent in the novel arm during the choice phase was calculated by the formula ([time spent in the novel arm]/[time spent in all arms]) × 100 and used as the index for short-term spatial recognition memory. In addition, total distance moved on the entire maze was recorded and analysed in order to assess general locomotor activity.

2.2.4. Social Interaction Test

We assessed social interaction using a social approach test in a modified Y-maze as established before (Richetto et al., 2015; Weber-Stadlbauer et al., 2017). The apparatus was identical to the one described above for spatial working memory. Again, the maze was elevated 90 cm above the floor and was positioned in a dimly-lit room (16 lx in the maze). For this test, two of the three arms contained rectangular wire grid cages (13 × 8 × 10 cm, length × width × height; bars horizontally and vertically spaced 9 mm apart). The third arm did not contain a metal wire cage and served as the start zone. The apparatus was located in an experimental testing room under dim diffused lighting (~35 lx as measured in the individual arms).

During the test, one wire cage contained an unfamiliar C57BL6 mouse, the other one contained a ‘dummy mouse’ made of black LEGO™ (Billund, Denmark). The allocation of the objects (live mouse versus dummy object) was counterbalanced across arms and treatments. At the beginning of a test session the animal was placed in the end of the start arm and allowed to explore freely for 5 min. A camera, mounted above the maze, captured images and transmitted them to a tracking system to assess general locomotor activity. Social interaction was defined as nose direction to the wire cage in a 6-cm interaction zone adjacent to the wire cage and was assessed by a trained experimenter blind to the treatments. After 5 min the animal was removed and the apparatus was cleaned. The percent time spent with the live mouse was calculated by the formula (time spent with the live mouse/(time spent with the live mouse + time spent with the dummy object)) × 100 and used to assess relative exploration time between a congenic mouse and an inanimate dummy object.

2.2.5. Prepulse inhibition of the acoustic startle reflex

Sensorimotor gating was assessed using the paradigm of prepulse inhibition (PPI) of the acoustic startle reflex. The apparatus consisted of four brightly-lit startle chambers (100 lx) for mice (SanDiego Instruments, San Diego, CA) as fully described elsewhere (Meyer et al., 2005). The animals were presented with a series of discrete trials comprising a mixture of four trial types. These included pulse-alone trials, prepulse-plus-pulse trials, prepulse-alone trials, and no-stimulus trials in which no discrete stimulus other than the constant background noise was presented. The pulse and prepulse stimuli used were in the form of a sudden elevation in broadband white noise level (sustaining for 40 and 20 ms, respectively) from the background (65 dBA). In all trials, three different intensities of pulse (100, 110, and 120 dBA) and three intensities of prepulse (71, 77, and 83 dBA, which corresponded to 6, 12 and 18 dBA above background, respectively) were used. The protocol used for the PPI test was extensively validated before (Vuillermot et al., 2010; Vuillermot et al., 2011; Pacheco-Lopez et al., 2013). The animals were placed into the Plexiglas enclosure and adapted to the apparatus for 2 min before the first trial began. The first six trials consisted of six startle-alone trials; such trials served to habituate and stabilize the animals' startle response and were not included in the analysis. Subsequently, the animals were presented with 10 blocks of discrete test trials. Each block consisted of (i) three pulse-alone trials (100, 110, or 120 dBA), (ii) three prepulse-alone trials (+6, +12, or +18 dBA above background), (iii) nine possible combinations of prepulse-plus-pulse trials (three levels of pulse and three levels of

Table 1
Sequences of forward and reverse primers and probes used in qRT-PCR analysis.

Gene	Forward primer	Reverse primer	Probe
CLOCK	5′-AAGTCATCTCACACCGCAGT-3′	5′-ACCTCCGCTGTGTCATCTTT-3′	5′-GATCCCTACTGATACTAGCACTCC-3′
BMAL1	5′-ACTCGCACATGGTTCCACAA-3′	5′-GTTCCTGTGGTAGATACGCCAA-3′	5′-CGGGTGAAATCTATGGAGTACG-3′
ZIF268	5′-AGCGCCTTCAATCCTCAAG-3′	5′-TTTGGCTGGGATAACTCGTC-3′	5′-CAACCCTATGAGCACCTGACCACA-3′
cFOS	5′-TCCTTACGGACTCCCCAC-3′	5′-CTCCGTTTCTCTTCCTCTTCAG-3′	5′-TGCTCTACTTTGCCCCTTCTGCC-3′
36b4	5′-AGATGCAGCAGATCCGCAT-3′	5′-GTTCTTGCCCATCAGCACC-3′	5′-CGCTCCGAGGGAAGGCCG-3′

prepulse), and (iv) one no-stimulus trials. The 16 discrete trials within each block were presented in a pseudorandom order, with a variable interval of 15 s on average (ranging from 10 to 20 s). For each of the three pulse intensities (100, 110, or 120 dBA) PPI was indexed by percent inhibition of the startle response obtained in the pulse-alone trials by the following expression: 100% ∗ (1-[mean reactivity on prepulse-plus-pulse trials/mean reactivity on pulse-alone trials]), for each animal, and at each of the three possible prepulse intensities (+6, +12, or +18 dBA above background).

2.2.6. Amphetamine sensitivity test

The amphetamine sensitivity test was conducted in 4 identical open-field arenas (40 × 40 × 35-cm high) made of white plexiglass. They were located in a testing room under lighting (approximately 28 lx as measured in the center of the arenas). A digital camera was mounted directly above the 4 arenas. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus, Wageningen, The Netherlands) tracking system to record locomotor activity indexed by the distance moved in the entire open field arena.

To acclimatize the animals to the open field, they were placed in the center of the arena and allowed to explore freely for 20 min. At the end of this time period, the animals were removed from the apparatus and injected intraperitoneally (i.p.) with saline (isotonic 0.9% NaCl) solution to ascertain possible group differences to acute stress exposure induced by the injection procedure. Following saline injection, the animals were immediately returned to the same arenas and allowed to explore for another 20 min. Subsequently, the animals were briefly removed from the apparatus once more, administered with AMPH, and returned to the same arenas again. The locomotor responses to the acute drug challenge were then monitored for a period of 90 min. D-amphetamine sulfate (Sigma-Aldrich) was dissolved in isotonic 0.9% NaCl solution to achieve the desired concentration for injection. AMPH was administered i.p. at a dose of 2.5 mg/kg based on previous findings (Meyer et al., 2008). The volume of injection was 5 ml/kg for all solutions. All solutions were freshly prepared on the day of testing.

In order to assess gene expression in response to amphetamine in light versus dark phase tested animals, an additional cohort of animals was exposed to a between-subjects amphetamine sensitivity test (data not shown) and sacrificed 30 min after amphetamine or saline injection.

2.3. Molecular Analyses

2.3.1. Collection of brain samples

Two cohorts of animals were used or the collection of brain samples. The first were killed immediately after the open field test, while the second were killed 30 min after amphetamine (or saline) injection. After decapitation, brains were immediately extracted from the skull, frozen on dry ice and stored at −80 °C until further processing. This was followed by preparing 1-mm coronal brain sections using razor-blade cuts and subsequent micro-dissection of the brain areas of interest. In particular, we dissected the medial prefrontal cortex (mPFC; bregma: +2.3 to +1.3 mm), hippocampus (HPC; bregma −2.1 to −3.0 mm), the nucleus accumbens (NAc) (bregma +1.3 to +0.6 mm) and the midbrain (Mid) (bregma −3.0 and −4.0).

2.3.2. RNA preparation

Total RNA was isolated using the RNeasy Plus Universal Mini Kit (Qiagen™). The procedure was conducted according to the manufacturer's instructions, and the resulting RNA was quantified by spectrophotometric NanoDrop analysis.

2.3.3. Gene expression analysis by quantitative real-time polymerase chain reaction

RNA was analysed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iTaq™ Universal Probes One-Step Kit for probes (Bio-Rad Laboratories). The samples were run in 384-well formats in triplicates as multiplexed reactions with a normalizing internal control. We chose 36B4 as internal standard for gene expression analyses. Thermal cycling was initiated with an incubation at 50 °C for 10 min (RNA retrotranscription) and then at 95 °C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process and then for 30 s at 60 °C for the annealing and extension reaction. Relative target gene expression was calculated according to the 2(−Delta Delta C(T)) method. The probe and primers sequences used are summarized in Table 1, except for TH (Mm00447557_m1), DAT (Mm00438388_m1), Arc (Mm01204954_g1), Per1 (Mm00501813_m1) and Per2 (Mm00478099_m1) that were purchased from Thermofisher Scientific (Switzerland).

2.4. Statistical analyses

In the open-field test, the total distance moved served as the critical test readout and was analysed using a 2 × 2 × 12 (sex × daylight phase × 5-min bins) repeated-measures ANOVA. In the Y-maze working memory test, the relative time spent in the novel arm and distance moved during the choice phase were analysed using a 2 × 2 (sex × daylight phase) ANOVA. In the social interaction test, the relative exploration time between an unfamiliar congenic mouse and an inanimate dummy object was analysed using a 2 × 2 (sex × daylight phase) ANOVA of the per cent interaction time. In the PPI test, per cent PPI (%PPI) was analysed using a 2 × 2 × 3 × 3 (sex × daylight phase × pulse level × prepulse level) repeated measures ANOVA. The reactivity to pulse-alone trials was analysed using a 2 × 2 × 3 (sex × daylight phase × pulse level) repeated measures ANOVA, and the reactivity to prepulse-alone trials using a 2 × 2 × 3 (sex × daylight phase × prepulse level) repeated measures ANOVA. In the test of AMPH sensitivity, the total distance moved during the initial habituation and saline phases was expressed as a function of 5-min bins and analysed using a 2 × 2 × 4 (sex × daylight phase × 5-min bins) repeated-measure ANOVA. Total distance moved during the 90-min AMPH phase was also expressed as a function of 5-min bins and analysed using a 2 × 2 × 18 (sex × daylight phase × 5-min bins) repeated-measure ANOVA. Statistical significance was set at $p < 0.05$. Behavioural testing's statistical analyses were performed using the statistical software StatView (version 5.0).

The neurochemical qPCR data were first analysed using Student's *t*-tests (two tailed), followed by Bonferroni correction to account for the multiple comparisons in each brain area. Gene expression analysis in the amphetamine treated cohort were analysed using a two-way

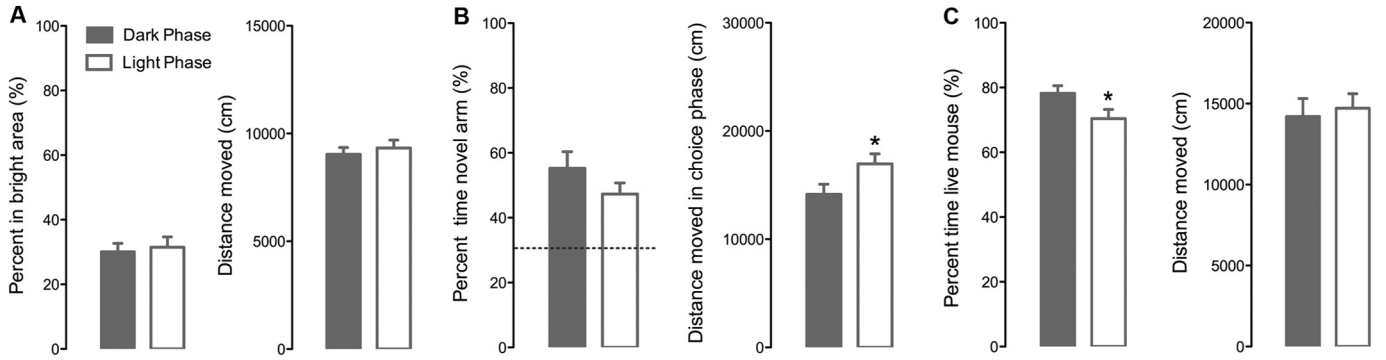

Fig. 1. Effects of light- versus dark-phase on anxiety-related behaviour, working memory in a Y-Maze and social behaviour. (A) Light-Dark-Box: The bar plots depict precent time spent in the bright compartment and distance moved (cm) in the light-dark box of anxiety-related behaviour. (B) Y-Maze-Test: The bar plots depicts the percent (%) time spent in the novel arm and total distance moved (cm) during the choice phase of a spatial working memory test in the Y-Maze. The dashed line indicates chance level (33%). (C) Social Interaction Test: Bar plots show the relative exploration time with an unfamiliar congenic mouse over an inanimate dummy for both groups, as well as the corresponding distances moved in the social interaction test. (A–C) N = 8–10 m and 8–10 f per group. *$p < 0.05$, reflecting the significant main effect of light-phase. All values are means ± SEM.

(daylight phase x drug treatment) ANOVA followed by LSD Post-hoc tests whenever appropriate. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software IBM SPSS Statistics (version 19).

3. Results

3.1. Effects of light and dark phase testing on anxiety, locomotor activity, spatial recognition memory and prepulse inhibition

We first evaluated the impact of dark versus light phase testing on innate anxiety-like behaviour using the LDB test. As shown in Fig. 1A, the animals expressed a natural aversion towards the brightly illuminated arena, regardless of the daylight phase during which the test was conducted. Indeed, we observed no difference between animals tested during the light phase and animals tested during the dark phase, which was also not confounded by effects on locomotor activity, as the distance moved during the test was similar in both groups. Given the lack of a sex x daylight interaction, male and female subjects are depicted together.

We next assessed spatial recognition memory in the Y-maze. As shown in Fig. 1B, mice tested in the light phase showed a trend for decreased, even though not significantly different, preference for the novel arm compared to animals tested in the dark phase. Male and female subjects are once again depicted together, given the lack of significant sex x daylight phase interaction. Interestingly, 2 × 2 (sex × daylight phase) ANOVA analysis of the total distance moved in the choice phase showed that mice tested during the light phase had higher locomotor activity ($p < 0.05$) when compared to animals tested during the dark phase suggesting a possible effect of testing time on locomotor activity.

In the social interaction test, the relative exploration time between an unfamiliar congenic mouse and an inanimate dummy object was used to index social interaction. As shown in Fig. 1C, mice tested in the light phase display a decreased preference for the congenic mouse ($p < 0.05$). Given the lack of a significant daylight x sex interaction, males and females are again depicted together. This effect was not confounded by changes in general locomotor activity (Fig. 1C). Hence, the light-phase-induced social interaction deficit reflects a selective impairment in social approach behaviour.

We furthermore assessed locomotor activity in the open field. The total distance moved in the entire arena was expressed as a function of 5-min bins and analysed using a 2 × 2 × 12 (sex × daylight phase × 5-min bins) repeated-measures ANOVA. As shown in Fig. 2A, animals from both groups displayed a clear habituation effect across the 1-h testing period (main effect of 5-min bins: $p < 0.001$). Interestingly, the overall total distance moved was significantly increased in mice tested in the light phase relative to animals tested in the dark phase (main effect of daylight phase: $p < 0.001$), as also depicted by the bar plot of mean distance moved during the whole test period ($p < 0.001$). We did observe a statistically significant effect of sex across both phases, with females having a higher locomotor activity ($p < 0.05$) when compared to male animals. However, we did not observe a sex x daylight phase interaction, thus Fig. 2A displays the general effect of testing time on locomotor activity in both sexes.

Lastly, the analysis of % PPI revealed that mice tested in the light phase display a significant overall reduction in % PPI compared to dark phase tested mice ($p < 0.05$) as summarized in Fig. 2B. The PPI-disrupting effects of light phase largely emerged independent of the precise pulse and prepulse stimuli used, i.e., the interactions between light phase and pulse or prepulse levels failed to reach statistical significance. As expected, the reactivity to prepulse-alone trials increased with increasing prepulse levels (main effect of prepulse levels: $p < 0.001$). This effect was not influenced by the light phase (data not shown). Furthermore, the light phase did also not affect the startle reaction to the distinct pulse used. Startle reactivity generally increased as a function of increasing pulse levels (main effect of pulse: $p < 0.001$), with this effect being highly similar in both groups (data not shown).

3.2. Effects of light and dark phase testing on immediate early genes expression levels

We next investigated the gene expression levels of the IEGs Arc, cFos and Zif268 in various brain regions relevant for the translational behavioural paradigms discussed previously. As shown in Fig. 3A and C, the gene expression levels of Arc and Zif 268 in response to behavioural manipulation did not vary according to the daylight phase, with constant levels of expression observed in the mPFC, HPC, NAc and Mid. On the other hand, as shown in Fig. 3B, we observed a significant increase in the mRNA levels of cFos in the NAc and Mid of animals tested during the light phase when compared to animals tested during the dark phase. This effect suggests that the degree neuronal activation in behaviourally tested animals, indexed as expression of cFos, is time-of-day dependent in these two brain regions.

3.3. Effects of light and dark phase testing on clock gene expression levels

We then went on to assess the gene expression levels of the clock genes Clock, Bmal1 Per1 and Per2. As shown in Fig. 4A, the gene

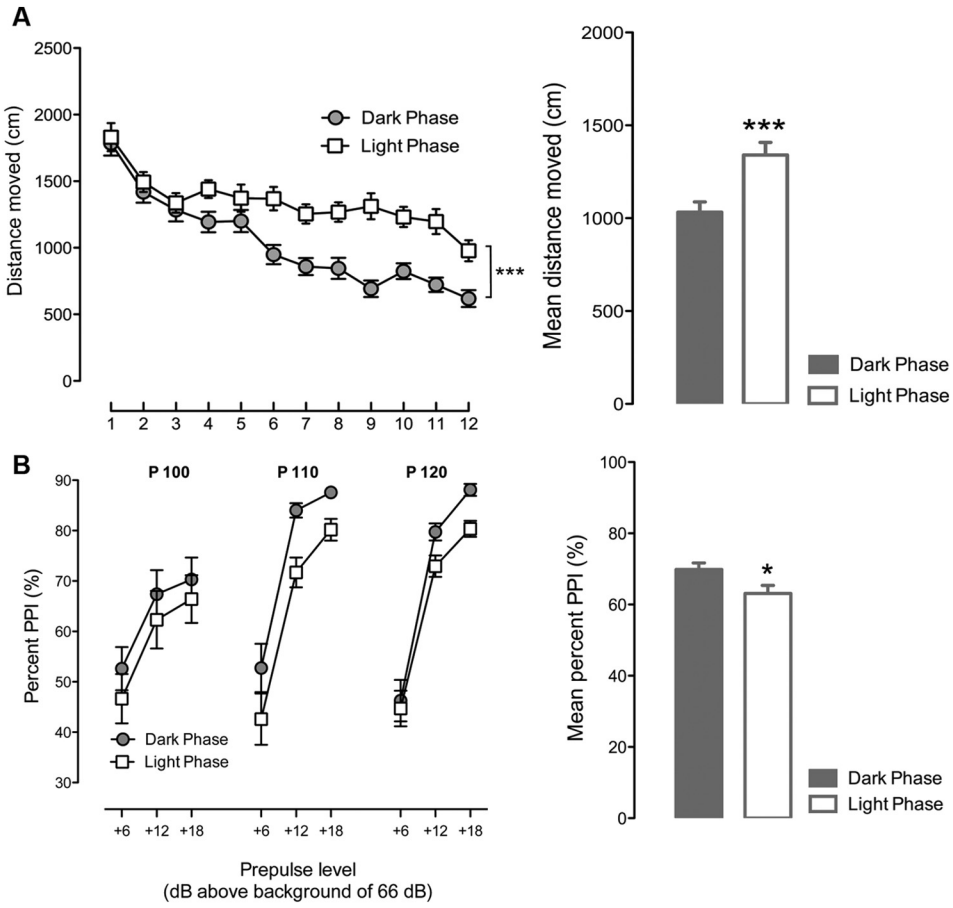

Fig. 2. Effects of light- and dark-phase on exploratory behaviour in the open field and on central information processing. (A) The line plots depict the total distance moved in the entire arena as a function of 5-min bins during the whole 1-h test phase in the open field and the bar plot depicts the means of these dependent measures across the entire test period. $N = 8–10$ m and 8–10 f per group. $^{***}p < 0.001$, reflecting the significant main effect of testing time. (B) The line plot shows percent prepulse inhibition (%PPI) as a function of different pulse and prepulse intensities in the test of PPI of the acoustic startle reflex. The bar plot depicts the mean %PPI across all pulse and prepulse levels tested. $N = 10$ m and 10 f per group. $^{*}p < 0.05$, reflecting the significant main effect of light-phase. All values are means ± SEM.

expression levels of Clock did not vary according to the daylight phase in the mPFC, HPC and NAc, while we observed significant increase in the Mid of light-phase tested animals ($p < 0.01$). Moreover, we observed that light-phase tested animals showed significantly increased Bmal1 gene expression levels compared to dark-phase tested animals in all the four brain areas analysed (Fig. 4B, $p < 0.001$ in the mPFC and HPC, $p < 0.01$ in the NAc and Mid), confirming a daily rhythmic expression of this gene in behaviourally tested animals that extends beyond the suprachiasmatic nucleus. When considering Per1, we observed a significant decrease of the expression levels of this gene only in the mPFC of light-phase tested animals (Fig. 4C, $p < 0.05$), while Per2 expression levels were strongly decreased in all the brain areas analysed (Fig. 4D, $p < 0.001$ in the mPFC, HPC, NAc and Mid). These results are consistent with the rhythmic transcription of the Period genes, which express a phase opposite that of Bmal1 (Reppert and Weaver, 2002).

3.4. Effects of light and dark phase testing on amphetamine sensitivity

We furthermore compared the sensitivity to the indirect dopamine receptor agonist Amph in light-phase versus dark-phase -tested animals. As expected, acute Amph administration at 2.5 mg/kg markedly increased locomotor activity as indexed by the distance moved in an open field (Fig. 4A). Compared to dark-phase tested animals, mice tested in the light phase displayed a significant increase in Amph-induced locomotor activity (main effect of light phase: $p < 0.01$; Fig. 4A), as also depicted by the bar plot of mean distance moved during the whole test period ($p < 0.01$). The two groups did not differ in terms of locomotor activity during the initial habituation phase, but light-phase-tested animals displayed a trend towards increased locomotor activity towards the end of the Sal administration phase (Fig. 4A, $p < 0.01$), similar to the previous findings in the open field (Fig. 1B). Again, data analysis revealed a significant main effect of sex ($p < 0.05$), but no interaction with the drug treatment, which is why the data are presented for male and female animals together. Together, these data demonstrate increased sensitivity of animals tested in the light-phase versus dark-phase to the psychostimulant drug Amph.

3.5. Effects of light and dark phase amphetamine sensitivity testing on dopaminergic gene expression levels

Lastly, we investigated the gene expression levels of DAT, TH and cFos in the NAc and Mid following the amphetamine sensitivity test performed during different daylight phases. Confirming our previous findings, cFos gene expression was affected by the daylight phase in both the NAc and Mid, where we observed a significant increase of Cfos gene expression levels in light phase-tested animals (Fig. 5B, main effect of daylight phase: $p < 0.01$) compared to dark phase-tested animals. On the other hand, AMPH treatment increased cFos gene expression in both groups only in the NAc (main effect of AMPH: $p < 0.001$), while no statistically significant effect was detected in the Mid.

When considering the transcription levels of DAT in the NAc, we observed both a main effect of APMH ($p < 0.05$) and an interaction between daylight phase and AMPH treatment ($p < 0.01$, Fig. 5B). Subsequent *post-hoc* tests revealed a decrease in DAT gene mRNA levels in light-phase tested animals when compared to animals tested in the dark-phase ($p < 0.01$), and a decrease in the DAT gene expression profile of dark-phase tested animals treated with AMPH compared to those treated with saline solution ($p < 0.01$). On the other hand, transcription levels of TH were significantly decreased by the AMPH treatment both in animals tested during the day and during the night (main effect of AMPH treatment: $p < 0.01$), while no effect of daylight

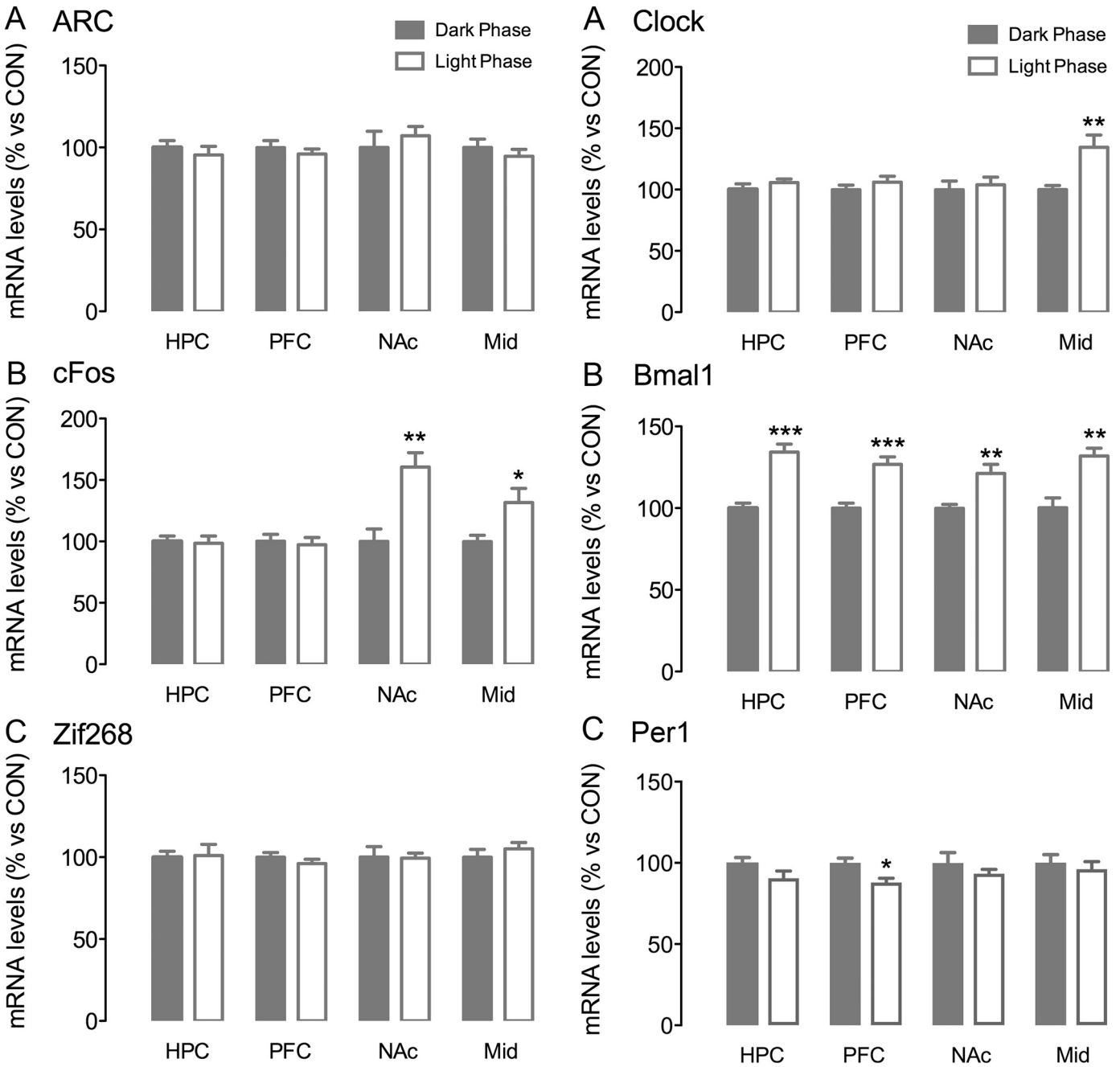

Fig. 3. Effects of light phase testing on ARC, cFos and Zif268 expression. The graphs depict the levels of normalized mRNA expression in the hippocampus (HPC), prefrontal cortex (PFC), Nucleus Accumbens (NAc) and midbrain (Mid) assessed using quantitative RT-PCR. (A) ARC gene expression in animals in light- versus dark-phase. (B) cFos gene expression in animals in light- versus dark-phase. *$p < 0.05$, **$p < 0.01$, reflecting the significant main effect of light phase. (C) Zif268 gene expression in animals in light- versus dark-phase. (A–C) $N = 6$ m and 6 f per group. All values are means ± SEM.

phase was detected (Fig. 5B).

Lastly, in the Mid, we observed similar expression patterns for both TH and DAT. In detail, the transcriptional profile of both genes was reduced in animals tested in the light-phase (main effect of daylight phase: $p < 0.05$), while the same trend failed to reach statistical significance in AMPH treated mice.

(caption on next page)

Fig. 4. Effects of light phase testing on Clock, Bmal1, Per1 and Per2 gene expression. The graphs depict the levels of normalized mRNA expression in the hippocampus (HPC), prefrontal cortex (PFC), Nucleus Accumbens (NAc) and midbrain (Mid) assessed using quantitative RT-PCR. (A) Clock gene expression in animals in light- versus dark-phase. (B) Bmal1 gene expression in animals in light- versus dark-phase. (C) Per1 gene expression in animals in light- versus dark-phase. (D) Per2 gene expression in animals in light- versus dark-phase. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, reflecting the significant main effect of light phase. (A–B) $N = 6$ m and 6 f per group. All values are means ± SEM.

4. Discussion

The present study investigated the impact of daylight phases on the behavioural profile of female and male C57BL/6N mice. Importantly, our data demonstrates that locomotor activity, social interaction, and PPI scores are significantly affected by the daylight phase, while the innate anxiety and spatial working memory are not altered, suggesting that the phase-associated differences in the behavioural performance are specific for certain neurobiological functions.

Specifically, we were able to demonstrate an effect of light-phase testing on locomotor activity, as the activity level in the open field was significantly increased in mice tested during the light versus the dark phase. As a variety of behavioural tests in mice critically depend, to a certain extent, on locomotor activity, the precise phase of day during which the test is conducted could therefore impact many different tests, even if the level of activity is not the primary readout of interest. Other groups have also reported a light-phase dependent difference in basal locomotor activity, with controversial results (Valentinuzzi et al., 2000; Hostetter, 1966). This controversy could potentially be explained considering the substantial body of literature showing that nocturnal rodents are able to adjust their activities also to non-photic entraining factors (Daily and Ehrlich, 1996; Mistlberger and Skene, 2004; Mrosovsky, 2003; Shkolnik, 1971). For laboratory mice, human activities in the vivarium and test facilities may actually represent such non-photic circadian entrainers. It is thus likely that results across laboratories can vary depending on whether animal husbandry requirements represent unavoidable disruptions to mice during their natural resting phase or whether animals are kept under a reversed light-dark cycle, thus having an undisturbed resting time during the light hours, and the routine husbandry activities happened during their active (resp. dark)

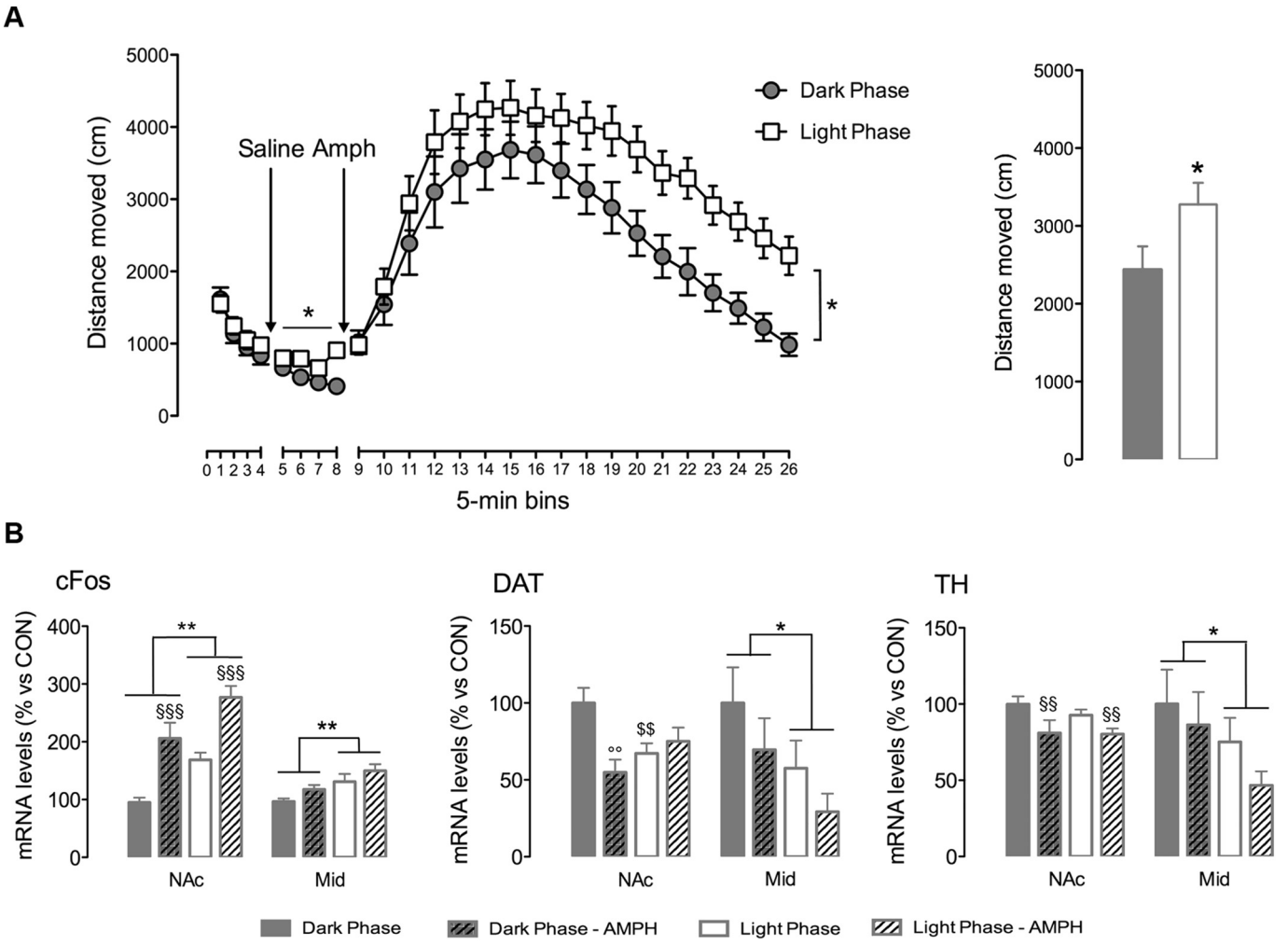

Fig. 5. Effects of light- and dark-phase and of an acute systemic amphetamine challenge on locomotor activity (A) and on cFos, DAT and TH expression in the Nucleus Accumbens (NAc) and midbrain (Mid) assessed using quantitative RT-PCR (B). (A) All animals were first placed into the open field and allowed to habituate for 20 min, after which they were first injected with saline and subsequently with Amphetamine (Amph; 2.5 mg/kg) solution. The line plot shows the distance moved as a function of 5-min bins during the initial habituation, the saline and the subsequent drug phase, and the bar plot depicts the mean distance moved during the drug phase. $^{*}p < 0.05$, $^{**}p < 0.01$, reflecting the significant main effects of light phase. $N = 10$ m and 10 f per group. (B) cFos, DAT and TH expression in animals after an acute systemic amphetamine challenge in light- versus dark-phase. * < 0.05, $^{**}p < 0.01$, reflecting a main effect of light phase; §§ $p < 0.01$, §§§ $p < 0.001$, reflecting a main effect of Amph; $^{\circ\circ}p < 0.01$, reflecting a significant post-hoc effect of Amph in the dark phase. $N = 6$ m and 6 f per group. All values are means ± SEM.

phase. Thus, it is important to consider potential effects of the animals' non-photic entrainment versus an actual light-induced circadian effect. Moreover, in our current setup, we cannot fully exclude the possibility that the exposure to light during the dark phase in the previous tests could affect the entrainment of the animals to the dark-light cycle, and thus modify their behaviour in subsequent tests. However, the Open field was the second test that we performed, and as the animals were allowed a four-day resting period between tests, we deem it highly unlikely that the previous exposure was sufficient to desynchronise the animals from their circadian rhythm.

Limited studies have also addressed the impact of circadian timing on PPI experiments, reporting, once again, contradictory results (Adams et al., 2008; Weiss et al., 1999). In the hope of clarifying this issue we thus assessed PPI performance during the light and dark phase. As described, we observed a significant decrease in PPI performance of mice tested during the light phase. This finding is crucial for preclinical research into behavioural neuroscience, especially with relevance to neuropsychiatric disorders, where sensorimotor gating is one of the most commonly tested behavioural domains (Geyer et al., 2001; Van den Buuse, 2010; Karl et al., 2011).

Likewise, time of testing also had a significant effect on sociability in mice: when tested during the light phase, animals spent less time interacting with a conspecific mouse. Similarly, social behaviour is commonly and widely assessed in several disease models and one of the critical readouts for models of autism and schizophrenia (Bitanihirwe et al., 2010; Meyer, 2014). Our findings, however, are partially in controversy to previously reported circadian patterns of social behaviour, where social approach behaviours were found to be similar throughout the daylight phases (Yang et al., 2008; Yang et al., 2007). We can only speculate about the reasons underlying these controversial findings, however, one important factor could relate to differences in housing conditions. Indeed, recent findings reported a drastic effect of housing conditions on behaviour in C57/Bl6 mice: Individually Ventilated Cages (IVC), used as holding cages for our animals throughout the study, were shown to have a critical impact on behavioural experiments and make it difficult to reliably compare behavioural studies coming from IVC-housed mice to studies conducted on mice kept in different settings. Social behaviours, in particular, are different if animals are held in IVC cages compared to animals held in filter-top systems (Logge et al., 2013). Nevertheless, our study uncovers important effects of daylight phase testing on social behaviour, which should definitely be considered when testing animals that are housed in similar conditions. This statement is especially relevant in light of the increasing use of IVC cages (Logge et al., 2013). Once again, we cannot fully exclude the effect of previous light exposure during the night on subsequent testing performance. However, should this be the case, it does not modify the main message of our findings. Indeed, behavioural testing per se will always require a disturbance and exposure to light (when conducted in the dark phase) or a disturbance of the animals' resting phase (when conducted during the light phase) that could affect the animals' circadian rhythms. What we would like to highlight is that, for exactly this reason, the timing of behavioural testing should be further standardised among laboratories to increase reproducibility.

Interestingly, we also report that the animals' performance in anxiety- and cognition-relevant tests, such as the light-dark-box or the Y-Maze test, was not affected by the light phase in which the testing occurred. This suggests that the timing of testing impacts behavioural readouts in a specific manner, and possibly only affects certain neurobiological functions, including sensorimotor gating, social behaviour and locomotor activity. We acknowledge, however, that our study does not cover all the behavioural paradigms currently used in behavioural research, and additional studies are needed to further clarify which behavioural performances are affected by the timing of the test.

Based on our observations at the behavioural level, we decided to investigate whether the differences between night and day performances were associated with differences in neuronal activation, indexed by the gene expression levels of the immediate early genes Arc, cFos and Zif268. This analysis was performed in brain areas that are crucial for behavioural performance and psychopathology (Nieoullon, 2002; Spanagel and Weiss, 1999; Kauer and Malenka, 2007), namely the PFC, the HPC, the Mid and NAc. Importantly, our choice to include the NAc and Mid was further supported by the observation that the behaviours we found to be affected by light versus dark phase testing are strongly dependent on dopaminergic brain regions, such as the VTA and its main output regions (Groenewegen, 2003; Ralph-Williams et al., 2003; Soderpalm and Ericson, 2013; Swerdlow et al., 1990; Tzschentke, 2001). Moreover, a variety of studies has demonstrated an important influence of circadian patterns on dopaminergic circuits (Landgraf et al., 2016a; Landgraf et al., 2016b; Baltazar et al., 2014; Verwey et al., 2016; Kim et al., 2017; Mendoza and Challet, 2014), which further support our findings of different dopaminergic-dependent behavioural performances during night and day testing. Of note, all gene expression analyses were performed in cohorts of animals that underwent only one behavioural test (i.e. the Open Field test or the Amphetamine sensitivity test), thus ruling out any possible confounding factor related to previous exposure to light during dark phase testing in previous tests.

Interestingly, while the expression levels of Arc and Zif268 were not affected in any of the brain areas we analysed, the expression of c-FOS was dependent on the light-phase in both the NAc and Mid, highlighting a potential involvement of differential neuronal activation in these brain regions in mediating the observed behavioural effects. Whereas circadian fluctuations in c-FOS expression in general has been previously described (Grassi-Zucconi et al., 1993; van der Veen et al., 2008), this is, to our knowledge, the first study to report that the expression of c-FOS in the NAc and midbrain in response to a behavioural test is different between light-phase and dark-phase tested animals. Of note, our study fails to ascertain whether these differences in c-FOS expression are due to differential dark/light c-FOS expression at baseline, or if they are due to differential activation processes that occur during light and dark phase testing. However, while further studies are necessary to answer this question, our results are a first suggestion towards the notion that neuronal activation following behavioural testing could be different in light versus dark phase-tested animals.

Our behavioural and molecular findings thus suggest a possible role of dopaminergic activity in mediating the differential behavioural performance of mice tested in the light- versus the dark-phase. This is further supported by the increased sensitivity to Amphetamine displayed by the light-phase-tested mice, and by the analysis of gene expression levels of the dopaminergic markers DAT and TH. Indeed, in the Mid, we observed that the transcriptional profile of both genes was reduced in animals tested in the light-phase, which could be consistent with an increased basal dopaminergic tone during the light phase.

This increased neuronal activity, especially in the VTA and its main projection site, the NAc, may be a relevant mechanism underlying the light-testing-induced hyperactivity and potentiation of dopaminergic drug sensitivity. Indeed, the midbrain-striatal pathway is known to be pivotal for mediating dopamine-dependent behaviours such as basal and drug-induced locomotor activity (Groenewegen, 2003). For example, the locomotor-enhancing effects of low doses of systemic Amph (around 2.5 mg/kg for mice) typically emerge because of increased dopamine transmission in the NAc (Creese and Iversen, 1975; Heidbreder and Feldon, 1998; Pijnenburg et al., 1976) and are abolished by selective lesions of the NAc (Mele et al., 1998a; Mele et al., 1998b). It would, therefore, be intriguing to further test whether the light testing-induced potentiation of dopaminergic drug sensitivity may be causally linked to altered neuronal activity in those pathways and allied neurotransmitter systems. Of note, we acknowledge that our interpretations are of speculative nature and would need to be confirmed by further analyses, such as measures of dopamine content and circuit activity at different times during the day.

Lastly, we observed that light-phase tested animals showed significantly increased Bmal1 gene expression levels, and reduced Per2

gene expression levels, compared to dark-phase tested animals in all the four brain areas analysed. This observation is in line with the rhythmic, and opposite, daily expression phases of Bmal1 and Per2 (Reppert and Weaver, 2002). However, the timing of this alternating expression seems to be in contrast with the classical expression pattern of these genes in the suprachiasmatic nucleus. Indeed, in this brain region, the expression levels of Bmal1 peak during the organism's subjective night (and of Per2 during the subjective day) (Pace-Schott and Hobson, 2002). However, other groups have also reported different clock gene expression profiles across extra-SCN brain regions, suggesting region specific brain clocks (Chun et al., 2015; Rath et al., 2013). Importantly, we acknowledge that our study is an incomplete suggestion in this direction, as we fail to report a complete pattern of expression at different time points. Moreover, it is important to mention that behavioural testing in our study, as well as in a great number of other laboratories, includes exposure to light (regardless of when the testing is conducted). This can either be a requirement of a certain test, as for example exposure to light to assess innate anxiety, or given by the technical setup of the laboratory (availability of light-independent tracking systems). Thus, in our study, it is not possible to fully disentangle the effects of light-exposure versus behavioural testing. While this is a clear limitation when it comes to the interpretation of potential circadian gene expression patterns, it does not influence the answer to the primary questions addressed in our work: Is the outcome of behavioural testing (under identical conditions) dependent on the time of testing and is this phenomenon paralleled by changes in certain gene expression patterns in brain regions, crucial for behavioural performance?

In summary, this is, to the best of our knowledge, the first study investigating the impact of daylight phases on such a wide variety of behavioural paradigms in male and female C57BL/6 mice. Our findings further suggest that alterations in circadian and dopaminergic gene expression in mesolimbic brain structures might be involved in the different behavioural responses of mice tested in the light- versus the dark-phase.

However, further research will be needed to directly ascertain whether altered neuronal activity within the mesolimbic dopamine system explains the effects of light-phase testing on (dopamine-related) behaviours.

In conclusion, our study adds to the growing body of evidence highlighting the critical importance of environmental factors, such as, for example, housing conditions (Logge et al., 2013), in the assessment and interpretation of behavioural phenotypes. Specifically, our findings demonstrate how performing behavioural experiments in different daylight phases can strongly affect the readout of a given test. The importance of these findings is heightened by the observation that this factor (i.e. phase of the light/dark cycle during which behavioural tests are conducted) varies greatly across laboratories, and its impact on the characterization of animal models is often not taken into account. As comparison and reproducibility of findings is pivotal in science, such factors should be addressed more carefully, and experimental protocols should be harmonised across different laboratories. Indeed, not taking this factor into account limits data reliability across generations of animal cohorts (when changes in light cycles occur) and across different research sites.

Ultimately, it is our strong belief that reproducibility across laboratories would greatly benefit from detailed descriptions and, as far as possible, standardization, of basic factors such as housing conditions (Logge et al., 2014) and the time or phase of behavioural testing.

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