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Differential entrainment of a social rhythm in adolescent mice

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Abstract

Daily routines in animal activities range from sleep-wake cycles, to foraging bouts, to social interactions. Among animals living within groups, it is unclear whether the motivations that underlie social interactions respond to daily light-dark (LD) cycles or endogenous circadian rhythms. Employing two mouse strains (BALB/cJ [BALB] & C57BL/6J [B6]) with genetically based differences in social affect and circadian rhythms, we examined how social investigation (SI) is modulated by social deprivation and circadian factors. We found a genetic influence on SI that was moderated by the preceding duration of social deprivation, requiring 3–6 hours of social isolation prior to testing. Following 6 hours of social deprivation, SI responses of adolescent B6 mice were greater than those of BALB mice only when the isolation period was imposed during the dark phase of the LD cycle. When B6 mice were weaned into conditions of constant darkness, a novel, endogenous social rhythm emerged, which was characterized by two pronounced peaks of social responsiveness (relative to one peak under LD entrainment) that were separated by 12-hour intervals. Irrespective of the lighting conditions during social isolation, the SI responses of adolescent BALB mice did not oscillate across the day. Similar strain-dependent patterns of sociability were evident within groups of mice that were left undisturbed in their home cage under LD entrainment or constant darkness. Overall, genetic influences on the social phenotypes of adolescent mice are thus moderated by an interaction between social deprivation and oscillations of an endogenous social rhythm that entrains to the LD cycle.

Keywords

social motivation; social neuroscience; chronobiology; circadian biology; circadian rhythm; behavior; *Mus musculus domesticus*

Introduction

A canonical view regarding the circadian biology of mammals is that a central pacemaker—situated in the superchiasmatic nucleus (SCN) of the hypothalamus—coordinates internal rhythms by integrating daily fluctuations in environmental cues (i.e., zeitgebers – *translation*

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from German, ‘time-giver’) with output pathways that modulate physiology and behavior [8, 31,50,55]. SCN output is typically monitored through behavioral variables, such as ambulatory or wheel-running activity [21], and physiological variables, such as body temperature, electroencephalographic activity and blood-glucocorticoid concentration [61]. Importantly, when animals are deprived of zeitgebers, such behavioral and physiological variables continue to oscillate with a near 24-hour period, indicating that an endogenous clock organizes their expression.

Recently, there has been a revived interest in elucidating the interplay between social factors and biological rhythms [13,16,34]. This resurgence was motivated in part by a series of studies demonstrating associations between psychosocial impairments and disturbances in the circadian rhythms of humans [8,18,39,44,49,62]. Social zeitgeber theory provides a framework for understanding these findings by postulating that social routines modulate biological rhythms and emotion-regulation processes [18], perhaps beginning in the womb [34]. Findings from animal studies support this view [13,19,20,30,60]. Another prediction based on this theory suggests that intrinsic variability in the functioning of a circadian pacemaker can lead to distinct patterns of social rhythm expression [34]. Diel rhythms in sociability have been identified among feral rodents, such as the elaborate morning-time ‘greetings’ of Olympic marmots [6], and in experimentally induced forms of aggression in laboratory rodents [52,58]. Moreover, distinct social rhythms have been reported for non-mammalian species [24,53]. Yet, the extent to which these social rhythms are associated with an endogenous pacemaker is unclear. Consistent with work in other systems [35,51], it is conceivable that social interactions may be responsive to a pacemaker located outside of the SCN. In this regard, it is important to note that access to motivating stimuli, such as food and psychostimulants, can entrain anticipatory locomotor activity in SCN-lesioned rodents [27,29]. Furthermore, pain sensitivity and positive affect, both of which oscillate in a circadian manner [7,9,11,22,37], may share common physiological mechanisms with some forms of sociability [40,41]. Thus, under conditions that promote high levels of social anticipation, social interactions may be highly responsive to circadian factors.

Comparative studies of BALB and B6 mice offer a rigorous model for exploring the rhythms of socio-emotional processes in the mammalian brain. Several studies indicate that genetic factors influence levels of sociability in BALB and B6 mice [36,42,54]. Moreover, output of the circadian pacemaker differs substantially between BALB and B6 mice, exhibiting a nearly 1-hour difference in endogenous period length (22.94 vs. 23.77 hours, respectively; see ref. [57]). Finally, early-adolescent B6 mice find social interactions highly rewarding while age-matched BALB mice are indifferent to similar social opportunities [43]. In the present experiments, we investigated relationships between circadian rhythms and social motivation in adolescent mice from these two strains.

Materials and Methods

Animal husbandry

BALB/cJ and C57BL/6J mice were purchased from the Jackson laboratories (Bar Harbor, ME) and subsequently bred at the University of Wisconsin (Madison, WI) under tightly controlled temperature ($21 \pm 1^\circ\text{C}$), humidity (50-60%) and lighting (white light, 200-300 lux; 14:10 h light/dark, ‘lights off’ from 1130-2130) conditions. Mice were maintained under specific-pathogen free conditions and housed in standard polypropylene cages ($290 \times 180 \times 130$ mm) with 1/8" grain-size corncob bedding (The Andersons), nesting material (Ancare Corporation), and *ad libitum* access to food (Teklad Rodent Diet, Harlan) and tap water. New mice were routinely introduced to the breeding colony and brother-sister matings were not conducted. Pregnant females were isolated at 10-15 days post-coitus and pups were weaned on postnatal day (PD) 20-21 (day of birth = PD 0) into social groups that contained 2 mice/sex from the same strain.

Two to 4 litters/strain were weaned at the same time, males and females were pooled into separate groups, and individuals were then selected randomly to generate a social group. For experiments involving constant darkness (see Results), social groups of weanling mice (PD 20/21), or newborn mice (<12 h from birth) and their mother, were transferred to an adjacent colony room illuminated with red light (10-20 lux). These cages were kept under opaque plastic covers, except during testing sessions and routine cage cleaning. All animal care and experimental protocols were conducted in accordance with the regulations of the institutional care and use committee at the University of Wisconsin-Madison and the NIH *Guide for the Care and Use of Laboratory Animals* (ISBN 0-309-05377-3). Personnel from our own laboratory conducted all aspects of the mouse husbandry under strict guidelines to insure gentle and consistent handling of the mice.

Social investigation tests

Mice were maintained undisturbed within their social group for 10-15 days after weaning. To reduce the number of independent variables associated with the different experiments, male test mice were always evaluated in response to female stimulus mice on PD 30-35. Importantly, previous studies demonstrate that the genotype of female stimulus mice does not influence the SI responses of male test mice at this stage of adolescent development [42]. Prior to testing, males (test mice) from a social group were isolated into a clean cage without nesting material, while their female cage mates (stimulus mice) remained in the home cage. Test mice were socially isolated for periods ranging from 15 min to 48 h. Test mice from each social group were isolated for the same amount of time. Cages containing the test and stimulus mice were transported from the mouse colony ≈ 5 m through a quiet and dimly lit (5-15 lux) intervening room ≈ 30 min prior to testing. All SI tests were conducted under dim red illumination (30-40 lux). Thus, for experiments in which mice were tested at a time that corresponded to the light phase of the LD cycle (see Results), mice were transported to the procedure room and tested under dim red illumination. For all data presented in Figures 1 and 2 of the Results, test mice were placed into a clean cage 15 min before testing began and the cage was covered with a transparent sheet of Plexiglas[®]. Testing was conducted in a clean cage to insure that between-groups variation in SI was influenced solely by social isolation, rather than by changes in test mouse behavior that may be associated with different durations of home cage residency. A familiar female member of the test mouse's social group was added to the cage of the test mouse, and social interactions were recorded with a 3CCD digital video camera (Canon GL2, Japan) for 5 min. All mice tested in the experiments that are presented in Figures 1 and 2 of the Results were evaluated for SI once. Recordings were transferred via firewire directly to a Dell[™] Pentium[®] IV desktop computer. SI responses were calculated as a composite of all measurements of pro-social behavior that a test mouse directed towards a stimulus mouse, as described in ref. [42]. The social behaviors of stimulus mice were noted, but not included in the measurement of SI. Instances of sexual-like behavior were very rare, occurring in <1% of all tests, and were not included in the composite measurement of SI.

For experiments presented in Figure 3 of the Results, each test mouse was evaluated twice and each test was separated by 36-48 h of social housing. Test mice were isolated into a clean cage 6 h before SI testing. For the experiments presented in Figures 3a-c of the Results, mice were tested either at zeitgeber time (ZT) 6 and ZT 18, or at ZT 12 and ZT 0/24. The order of SI testing (early/late or late/early; 'early' - ZT 6 or ZT 12 or 'late' - ZT 18 or ZT 0/24) was pseudo-randomized and counter-balanced across all experimental groups. The correlation value between the first and second SI test was low (Pearson's correlation, $r_p = -0.06$, $P=0.47$), indicating that social responsiveness of test mice did not habituate across testing sessions. For the experiments presented in Figures 3d-f of the Results, mice were tested at two times separated by 48 h of social housing, but both testing sessions were conducted at the same ZT. The lighting conditions used for LD entrainment and the isolation period prior to the first SI

test in these experiments were similar, but were alternated for the isolation period preceding the second SI test. All SI tests were conducted under dim red illumination (30-40 lux) and followed the experimental procedure described above.

Home cage observations

Cages of adolescent (PD 30-35) mice (2 mice/sex) were transported to a procedure room with entrainment conditions that were identical to the environment they were maintained in since weaning. Mice were then videotaped continuously for 24 h. Behavioral categories were assigned from an ethogram to the entire group of mice and scored continuously for a 10-min period during each hour of a 24-h recording session.

Behavioral categories were assigned to the majority of mice (3-4 individuals) within a social group for measures of locomotor activity, huddling and behavioral quiescence (see below). Behavioral transitions were noted only when a majority of the mice (>2 individuals) within the cage transitioned to a different behavioral state. *Ambulatory activity* was tallied when mice were actively moving and were distributed around the cage. *Behavioral quiescence* was scored when mice were clustered into a group, typically in the nest area, and individuals were not moving. *Social behavior* was scored when a mouse sniffed, groomed, followed another mouse, or pulled at the tail of a conspecific. *Huddling* (mice clustered into a group, but moving), *sexual behavior* and *feeding* were also scored. For instances when at least one mouse was engaged in a behavioral activity (e.g., ambulatory activity) that was distinct from others in the group (e.g., behavioral quiescence), a *mixed* behavior category was scored. Data for huddling, sexual behavior, feeding and the mixed behavior category are not presented in the Results section, but are available from the corresponding authors upon request. To help visualize trends in the 24-h observational data, all values were smoothed with the following function: $(x_{T-1} + x_T + x_{T+1})/3$, where x = the total duration a social group was engaged in a behavioral category during a single sampling period, T . One-third of all sampling periods were scored in duplicate by 2 different raters (inter-rater reliabilities for each behavioral category are provided in the legend of Fig. 4 in the Results section).

Behavioral analysis and statistics

Behaviors were scored with computer-assisted software (ButtonBox v.5.1, Behavioral Research Solutions). All SI tests were analyzed in duplicate, once during testing and once by a second, blinded rater from computer-stored videos. Inter-rater reliability was high (inter-rater reliabilities for each experiment are provided in the figure legends). All statistical analyses and graphical representations of SI are based on the average of these 2 measurements. Two- and 3-factor ANOVAs were used to assess SI and the behavioral categories from the ethogram, with the genotype of mice, isolation duration, entrainment condition or test time as between-group factors. Post-hoc comparisons were conducted with planned orthogonal comparisons that were controlled for type I error.

Results

In an initial set of experiments, the SI responses of adolescent (PD 30-35) male mice towards a former female cage mate were evaluated after a period of social isolation that ranged from 15 min to 48 h. Prior to each SI test, male mice were isolated into a clean cage and maintained under light-dark (LD) conditions according to the entrainment schedule that they were accustomed to since birth (14:10 h, light/dark; see the bottom of Fig. 1a). All individuals were tested at ZT 22, two hours before the onset of the light phase of the LD cycle. The SI responses of test mice were sensitive to a broad range of isolation periods (Figs. 1a & 1b; main effect of isolation duration, $P < 0.0001$). As the duration of social isolation was increased from 15 min to 3 h, BALB and B6 mice expressed comparable increases in SI (Fig. 1a; orthogonal contrast,

$P=0.10$). However, after 6 h of social isolation, B6 test mice exhibited higher levels of SI than BALB mice (Fig. 1a; orthogonal contrast, $P<0.001$). As the social isolation period was increased from 12 to 24 h, neither strain exhibited an associated increase in SI (orthogonal contrasts, 12-h vs. 24-h isolation periods for BALB [$P=0.20$] and B6 mice [$P=0.41$]). After 28-48 h of isolation, the SI responses of B6 mice, but not BALB mice, increased again (orthogonal contrasts, 24 h vs. longer isolation periods for BALB [$P=0.10$] and B6 mice [$P<0.0001$]). The association between the duration of social isolation and the SI phenotype of adolescent mice could be approximated with log-linear regression functions (Fig. 1b).

Increases in the SI response of B6 mice were most evident if the preceding social isolation period transpired during the dark phase of the LD cycle (Fig. 1a). For example, the increase in SI that unfolded across the first 3 h of social isolation for mice from both strains was associated with the dark phase, and the first manifestation of a strain difference in SI occurred after 6 h of social isolation in the dark (genotype $\times$ isolation duration interaction, $P<0.0001$). More subtle changes in SI were discernable after 9-24 h of isolation, concurrent with periods of social deprivation that occurred primarily during the light phase. The SI responses of B6 test mice increased markedly again after 28 h of social isolation, following 4 additional hours of isolation in the dark. Overall, the SI responses of B6 mice were thus particularly sensitive to 3-6 h of social isolation during *the dark phase of a single LD cycle*, with an additional increase after approximately 4 more hours of isolation during *the dark phase of a second LD cycle*.

To further examine how the lighting conditions during social isolation influenced the expression of the SI phenotype, test mice were examined at two time-points: ZT 14 (after 6 h of isolation in the light) or ZT 22 (after 6 h of isolation in the dark). At ZT 14, B6 mice expressed diminished SI responses relative to their responses at ZT 22 (Fig. 2; genotype $\times$ test time interaction, $P=0.09$; orthogonal contrast, $P=0.01$). Following isolation in the light from ZT 8-14, the SI phenotypes of BALB and B6 test mice were in fact indistinguishable (orthogonal contrast, $P=0.69$). To control for the possibility that the time of SI testing (at ZT 14) had itself diminished the SI responses of B6 mice relative to testing at ZT 22, test mice were evaluated at both time points after 24 h of isolation. Following a full day of social isolation, B6 mice expressed elevated SI responses relative to those of BALB mice, irrespective of test time (Fig. 2; genotype $\times$ test time $\times$ isolation duration interaction, $P=0.05$; orthogonal contrast, $P<0.0001$).

To examine whether these strain-dependent differences in SI oscillated throughout the day, BALB and B6 mice were tested following 6 h of social isolation at 6-h intervals across the 24-h LD cycle. At ZT 6 and ZT 12, when SI tests followed 6 h of social isolation during the light phase, the SI responses of BALB and B6 mice were of similar magnitude (Fig. 3a). When mice were assessed at ZT 18 and ZT 0/24, following isolation during the dark phase (for 4 and 6 h, respectively), the SI responses of B6 test mice were substantially higher than those of BALB mice (Fig. 3a; genotype $\times$ test time interaction, $P=0.008$). Therefore, the SI phenotypes of adolescent B6 and BALB mice were distinguishable only when social deprivation occurred during the dark phase of the LD cycle, regardless of the test time.

To determine whether the daily oscillation of SI among B6 mice (Fig. 3a) was associated with an endogenous rhythm, SI tests were conducted at 6-h intervals following 6 h of social isolation with mice that were maintained in constant darkness from the time they were weaned. Surprisingly, under constant darkness, B6 mice exhibited two distinct peaks, or acrophases, of social responsiveness that were separated by 12-h intervals (Fig. 3b; genotype $\times$ test time $\times$ LD entrainment interaction, $P<0.05$). In contrast to the unimodal pattern of SI expression under LD entrainment (Fig. 3a), constant darkness engendered a doubling of the SI acrophase frequency in B6 mice. The two B6 acrophases under free-running conditions were of similar magnitude to the single acrophase that occurred under LD entrainment (Figs. 3a & 3b,

orthogonal contrast, $P=0.35$). The SI responses of BALB mice housed in constant darkness were insensitive to the testing time (Fig. 3b; orthogonal contrast, $P=0.49$), similar to the invariant responses of BALB mice that were entrained to LD cues (Fig. 3a; orthogonal contrast, $P=0.46$).

To assess whether the bimodal SI response pattern of B6 mice housed under constant darkness was influenced by their pre-weaning experience ([45]; also see ref. [21]), the SI responses of test mice deprived of LD entrainment from birth were evaluated. Within this context, B6 mice no longer expressed circadian oscillations in SI expression (orthogonal contrast, $P=0.95$), even though their SI responses remained consistently higher than those of BALB mice at all test times (Fig. 3c; orthogonal contrast, $P<0.0001$). Thus, the bimodal SI response pattern of adolescent B6 mice maintained under constant darkness (Fig. 3b) required entrainment to periodic LD cues prior to weaning. By contrast, the SI responses of BALB mice remained arrhythmic when born into constant darkness, with no effect of entrainment history (orthogonal contrast, $P=0.49$) or time of testing (orthogonal contrast, $P=0.94$).

A series of control experiments were conducted to examine how the SI responses of adolescent B6 mice respond to an uncoupling of LD conditions from their circadian rhythms during social isolation. When BALB and B6 mice were tested at ZT 0/24, under 'phase-advanced' conditions (e.g., lights turned on during a normally dark 6-h isolation period), the elevated SI responses of B6 mice were reduced to levels comparable to those of BALB mice (Fig. 3d; genotype $\times$ isolation condition interaction, $P<0.05$). Following a 'phase-delayed' social isolation period, which entailed a prolonged dark phase for 6 additional hours with testing at ZT 6, the SI responses of BALB and B6 mice remained comparable (Fig. 3e; genotype $\times$ isolation condition interaction, $P=0.97$). In a third experiment, the elevated SI responses of B6 mice (relative to BALB mice) maintained in constant darkness were suppressed by 6 h of social isolation in the light (Fig. 3f; genotype $\times$ isolation condition interaction, $P<0.001$). Overall, these data demonstrate that the heightened SI responses of adolescent B6 mice required social isolation in the dark according to the expected circadian timeframe.

In a final set of experiments, the sensitivity of B6 sociability to circadian influences was evaluated within the home cage environment. The behaviors of adolescent BALB and B6 mice housed in mixed-sex groups, and maintained under a 14:10 h LD cycle or constant darkness (from weaning) were compared. Behavioral measurements were collected during regularly spaced 10-min sampling periods for each hour of a 24-h period. When mice were maintained under conditions of constant darkness for 10-15 days post-weaning, onsets of ambulatory activity occurred several hours prior to those of mice that were entrained to LD cues (Figs. 4c & 4d; ZT 12.5 and 13 for BALB and B6 mice entrained to LD cues, respectively, vs. ZT 4 and 8.5 for constant darkness), and there was an inverse relationship between measures of locomotor activity and behavioral quiescence across entrainment conditions (Figs. 4a-d; $r_p = -0.85$ and -0.78 for BALB and B6 mice, respectively). Moreover, under LD entrainment, BALB and B6 mice expressed 3-4 fold increases in their levels of social interaction at the transition from the light to the dark phase of the LD cycle (Figs. 4e-f; main effect of time, $P<0.05$; acrophases for BALB and B6 mice were ZT 17 and 19, respectively). Under both sets of housing conditions, B6 mice exhibited higher levels of social behavior than BALB mice (main effect of genotype, $P<0.0001$). Finally, B6 mice expressed 2 peaks in social behavior (at ZT 9 and 21) when they were maintained in constant darkness (Fig. 4f), consistent with the bimodal pattern of social responsiveness expressed by comparably housed B6 mice that were evaluated for SI (Fig. 3b). A correlational analysis revealed positive associations between ambulatory activity and social behavior for adolescent mice from both strains when they were entrained to a LD cycle ($r_p = 0.47$ and 0.58 for BALB and B6 mice, respectively). However, there was no relationship between ambulatory activity and social behavior when mice were maintained

in constant darkness ($r_p = 0.10$ and 0.03 for BALB and B6 mice, respectively), which indicates that locomotor arousal and sociability in adolescent mice are dissociable.

Discussion

At a proximate level, one factor that drives social approach among young mice is the rewarding experience of an ensuing social encounter [42,43]. Consistent with classical theories of reward [17,56,65], depriving an adolescent B6 mouse of social experience enhances its approach towards conspecifics [42] and the reward value that it derives from social reunion [43]. Thus, it appears that social isolation amplifies the sensitivity of reward circuits in the brain to social interactions [40]. Our current set of findings are consistent with the proposal that social motivation in young B6 mice is tightly coupled to the duration of social isolation, but it extends this relationship to incorporate the time when social deprivation occurs relative to the circadian cycle.

Under LD entrainment, the SI responses of B6 mice after 6 hours of social deprivation were greater following isolation periods that transpired during the dark phase of the LD cycle. One psychological explanation of this effect is that social isolation may be more salient during the dark phase of the LD cycle, a period when mice are generally awake and active [47,59]. Perhaps the experience of social isolation is dampened during sleep. Another possibility, not necessarily independent of the first explanation, is that isolation-induced effects on sociability among B6 mice are particularly sensitive to an endogenous oscillator. In support of this possibility, the SI responses of adolescent B6 mice that were maintained in constant darkness from weaning fluctuated rhythmically across a 24-hour period, indicating that some aspects of social behavior may themselves be *outputs* of a clock-based mechanism [19,64]. The social rhythm of adolescent B6 mice maintained under free-running conditions (i.e., constant darkness from weaning) cycled approximately two-times faster than their respective rhythm under LD entrainment. Thus, the B6 social rhythm is responsive to both an endogenous oscillator and LD entrainment. We do not yet know whether this behavioral rhythm in adolescent mice is specific to social interaction or whether it reflects a more generalized influence on motivational processes.

There is considerable evidence that social encounters can serve as an essential zeitgeber (see refs. [13, 34] for a review). For instance, in situations where periodic changes in LD conditions are not detectable, such as among bats deep inside caves [32] or root voles living under snow cover [26], individuals coordinate their behavior with the activities of other group members (also see refs. [13, 30]). Our present data show that the sociability of adolescent B6 mice oscillates in the absence of LD cues, which indicates that some motivational processes underlying social behavior may be driven by an endogenous circadian clock. However, the bi-acrophasic social rhythm of free-running B6 mice requires prior experience with periodic LD cues. Moreover, experiments in which the B6 social rhythm was desynchronized from the LD conditions demonstrated that light exposure during social isolation has a direct suppressive effect on the subsequent expression of SI. This disruption of the B6 social rhythm bears similarity to the re-setting influence of light in more traditional circadian paradigms. Overall, our work suggests that synchronized social activity may result from a group consensus of varied, individual endogenous rhythms, analogous to the averaging effect of early and late risers when breaking camp (e.g., see refs [12, 64])

The use of social isolation to assess SI precludes more orthodox measurements of circadian function, such as calculating period length and phase angles of entrainment, which require continuous monitoring across several days. However, adolescent BALB and B6 mice living undisturbed within their home cage expressed rhythmical patterns of social behavior similar to their respective SI phenotypes after social isolation. BALB and B6 mice expressed more

social behavior during the dark phase of the LD cycle, consistent with reports of a positive association between locomotor and social activity in house mice [5]. Furthermore, when B6 mice were housed in constant darkness, they exhibited bimodal patterns of social behavior, whereas BALB social behavior remained unimodal. From this perspective, it is important to consider whether circadian factors exert independent influences on overall levels of social activity versus the underlying motivations for sociability. For instance, although many rodents are active during the dark phase of the LD cycle [47,59], the social responsiveness of rodents after only a brief (e.g., 20-25 minutes) period of social deprivation appears to be insensitive to changes in LD conditions [48,63]. After longer periods of social isolation, however, LD cues exert substantial effects on the social responses of both mice (this study) and rats [38]. Taken together, these findings indicate that oscillations in rodent social activity in response to LD entrainment may be confounded, or 'masked', by rhythms in ambulatory activity under standard housing conditions (see ref. [21]). Moreover, although social rhythms may be diminished by certain external factors (e.g., social novelty or recent handling), they are readily identifiable within highly motivating contexts (e.g., SI tests that employ >3 hours of social deprivation). Critically, the relationship between LD entrainment and sociability may also depend on the age ([42,63]; also see refs. [22,25]) or the genetic background of the animal.

One particularly intriguing implication that emerges from these studies is the possibility that the reward value of social encounters may oscillate as a function of the circadian cycle. This notion is consistent with human accounts of circadian fluctuations in positive affect [7,11,37,44], a dimension of subjective experience that is closely tied to indices of sociability [14]. Interestingly, there have been several reports of diel oscillations in rodent behaviors which are associated with motivational processes, such as conditioned place preference responses mediated by psychostimulants [1,28], intracranial self-stimulation [46], opiate-mediated feeding [23], shock induced fear conditioning [10] and avoidance learning [15]. Moreover, accumulating evidence from studies of vertebrate and invertebrate taxa indicates that the molecular mechanisms that drive circadian rhythms also modulate behavioral and neuronal responsiveness to drugs of abuse [1-4,33].

Although BALB mice were active during the dark phase of the LD cycle, their SI responses were not differentially enhanced after social isolation in the dark versus the light phase of the LD cycle. The isolation-induced social responsiveness of BALB mice was also insensitive to free-running conditions (e.g., constant darkness from weaning or birth). Strain-dependent differences in the expression of SI therefore resulted from a temporally regulated change in the social responsiveness of adolescent B6 mice across the circadian cycle. Thus, in principle, it is reasonable to consider whether the central pacemaker located within the SCN of the hypothalamus may be functionally decoupled from the neural substrates that mediate social motivation in BALB mice. Alternatively, the BALB genetic background may impose more direct constraints on the neural circuits that underlie social rhythms. Nevertheless, from a broader perspective, our present findings represent a practical model for laboratory studies that aim to elucidate the underlying mechanisms through which systematic changes in zeitgebers moderate the expression of genetically influenced social traits.

Acknowledgements

With great sadness, we dedicate this paper to our friend, teacher and colleague Professor Ann E. Kelley (1954-2007). Ann's seminal contributions to understanding the integrated neurobiological processes underlying instrumental learning and motivation made her a pioneer in behavioral neuroscience. While her life was cut short, Ann's enormous zest and rigor for Science continues to flourish within the lives of those that she touched.

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Figure 1. Social investigation responses plotted as a function of the genotype of test mice and the preceding social isolation period

(a) SI responses are depicted above the abscissa. The duration and LD conditions of the respective social isolation periods are represented graphically below the abscissa. Sample sizes for the BALB and B6 genotypes were 6-15 and 10-24 test mice/isolation duration, respectively. Each asterisk represents a significant post-hoc comparison between the 2 genotypes (Tukey's HSD test, $P < 0.05$). (b) Log-linear regression functions were used to summarize the relationship between SI and social isolation for each genotype. The shaded area bounding each regression line represents the 95% confidence interval of the respective fit. Inter-rater reliability, $r_p = 0.94$, d.f. = 275. All data are presented as the mean $\pm$ standard error.

Figure 2. Social investigation responses of adolescent mice plotted as function of genotype, isolation duration and test time

The SI responses of adolescent mice were evaluated at ZT 14 or ZT 22 following either 6 or 24 h of social isolation. Data for ZT 22 are re-plotted from Figure 1a. Sample sizes for ZT 14 were 18-24 and 12-22 mice/genotype for the 6 h- and 24 h-isolation periods, respectively. Sample sizes for ZT 22 were 14-21 and 15-24 mice/genotype for the 6-h and 24 h-isolation periods, respectively. Asterisks represent a significant ($P < 0.05$) orthogonal contrast between the 2 genotypes. Inter-rater reliability, $r_p = 0.88$, d.f. = 147. All data are presented as the mean $\pm$ standard error.

Figure 3. Social investigation responses of adolescent mice maintained under different entrainment schedules and following circadian rhythm disruption

The SI responses of mice (a) entrained to a 14:10 h LD cycle (b) weaned into constant darkness and (c) maintained in constant darkness from birth. All SI tests were conducted at 6-h intervals following 6 h of social isolation. At each test time, respective sample sizes for the BALB and B6 strains were (a) 14-16 and 16-18 mice, (b) 13-16 and 16 mice, and (c) 6-8 and 10-12 mice. Each asterisk represents a significant ($P < 0.05$) orthogonal contrast between the 2 genotypes. Inter-rater reliability for Figures 3a-c, $r_p = 0.91$, d.f. = 309. The SI responses of mice were also measured after a 6-h phase shift. Mice were entrained to a 14:10 h LD cycle and tested at (d) ZT 0/24 following 6 h of social isolation in the dark ('Iso. dark') or in the light ('Phase advance'), and at (e) ZT 6 following 6 h of social isolation in the light ('Iso. light') or in the dark ('Phase delay'). (f) Mice were maintained in constant darkness from weaning and tested at CT 0/24 following 6 h of social isolation in the dark ('Iso. dark') or in the light ('Light pulse'). Respective sample sizes for BALB and B6 strains for each isolation condition were (d) 10 and 8 mice, (e) 6 and 8 mice and (f) 8 and 8 mice. Each asterisk represents a significant post-hoc comparison (Tukey's HSD test, $P < 0.05$) between the 2 genotypes. Numeric symbols represent a significant difference (Tukey's HSD test, $P < 0.05$) between B6 mice that underwent different LD conditions during social isolation. Inter-rater reliability for Figures 3d-f, $r_p = 0.85$, d.f. = 93. All data are presented as the mean $\pm$ standard error.

Figure 4. Comparisons of mouse activity patterns and social behaviors within their home cage environment

Behaviors of BALB mice (*left panels*) and B6 mice (*right panels*) maintained under a 14:10 h LD cycle (*stippled landscapes*) or under constant darkness (*black landscapes*) were compared. Behavioral measures include (**a,b**) behavioral quiescence, (**c,d**) ambulatory activity and (**e,f**) social behavior. The abscissa represents zeitgeber/circadian time. Samples sizes were 3-5 social groups/genotype for each zeitgeber condition. Measures of inter-rater reliability were high for behavioral quiescence ($r_p = 0.95$), ambulatory activity ($r_p = 0.91$) and social behavior ($r_p = 0.82$). Standard error bars (pooled across the entire 24-h cycle) are presented for each genotype and behavioral category at the top right of each panel.