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Circadian arrhythmia dysregulates emotional behaviors in aged Siberian hamsters

Brian J. Prendergast^{1,2}, Kenneth G. Onishi¹, Priyesh N. Patel³, and Tyler J. Stevenson^{1,4}

¹Department of Psychology, University of Chicago, Chicago, IL, USA, 60637

²Committee on Neurobiology, University of Chicago, Chicago, IL, USA, 60637

³School of Medicine and Public Health, University of Wisconsin, Madison, WI, USA, 53705

⁴Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK, AB24 3FX

Abstract

Emotional behaviors are influenced by the circadian timing system. Circadian disruptions are associated with depressive-like symptoms in clinical and preclinical populations. Circadian rhythm robustness declines markedly with aging and may contribute to susceptibility to emotional dysregulation in aged individuals. The present experiments used a model of chronic circadian arrhythmia generated noninvasively, via a series of circadian-disruptive light treatments, to investigate interactions between circadian desynchrony and aging on depressive- and anxiety-like behaviors, and on limbic neuroinflammatory gene expression that has been linked with emotionality. We also examined whether a social manipulation (group housing) would attenuate effects of arrhythmia on emotionality. In aged (14–18 months of age) male Siberian hamsters, circadian arrhythmia increased behavioral despair and decreased social motivation, but decreased exploratory anxiety. These effects were not evident in younger (5–9 months of age) hamsters. Social housing (3–5 hamsters/cage) abolished the effects of circadian arrhythmia on emotionality. Circadian arrhythmia alone was without effect on hippocampal or cortical interleukin-1 β (*IL-1 β*) and indoleamine 2,3-dioxygenase (*Ido*) mRNA expression in aged hamsters, but social housing decreased hippocampal *IL-1 β* and *Ido* mRNAs. The data demonstrate that circadian disruption can negatively impact affective state, and that this effect is pronounced in older individuals. Although clear associations between circadian arrhythmia and constitutive limbic proinflammatory activity were not evident, the present data suggest that social housing markedly inhibits constitutive hippocampal *IL-1 β* and *Ido* activity, which may contribute to the ameliorating effects of social housing on a number of emotional behaviors.

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Corresponding author: Brian J. Prendergast, Department of Psychology, Institute for Mind and Biology, Biopsychological Sciences Building, University of Chicago, 940 E. 57th St. Chicago, IL 60637 USA, prendergast@uchicago.edu, phone: (773) 702-2895, fax: (773) 702-6898.

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Keywords

emotionality; affective state; neuroinflammation; IL-1 β ; social

1. Introduction

Circadian rhythms (CRs) are endogenously-generated changes in physiology that give rise to coordinated daily patterns of cellular activity, hormone secretion, central nervous system (CNS) function and behavior [1]. Circadian clocks permit synchrony of the internal milieu and also allow physiological anticipation of predictable changes in the environment [2]. On a daily basis, the circadian pacemaker in the hypothalamic suprachiasmatic nucleus (SCN) entrains to the environmental light:dark cycle. A conspicuous CR driven by the SCN is the activity/rest cycle, but scores of physiological processes proceed in a circadian manner, including metabolism, neuroendocrine function, cognition, and diverse behaviors [3,4].

Affective processes are also under circadian control. Indeed, CRs in mood and emotional behavior are evident in species ranging from humans [5,6] to rodents [7]. The influence of the circadian system on affective regulation goes beyond merely providing temporal phase information, however, as the robustness of the circadian system also impacts emotional state. For example, disruptions in sleep-wake cycles and circadian rhythm abnormalities are significant risk factors for the emergence of psychiatric disorders such as depression and bipolar disorder in adolescents [8], and chronic jet lag is a significant risk factor in psychotic and major affective disorder relapse [9]. In patients with bipolar depression, therapies that involve circadian stabilization of social routines (scheduled sleep and daytime activity) have both remediative and prophylactic effects [10,11]. Polymorphisms in numerous circadian clock genes have also been associated with bipolar disorder and major depression in human populations (e.g., [12,13]). In preclinical models, mice with genome-wide mutations in core circadian clock genes express diverse changes in affective behavior. For example, *Clock*¹⁹ mutants exhibit emotional behaviors mimicking those of bipolar disorder (novel environment hyperactivity, reduced anxiety in the open field and light-dark tests, reduced depressive-like behavior in the forced swim test [14,15]). *Per2*^{*Brdm1*} mutant mice exhibit a similar resistance to depressive-like behavior in despair tests [16]. *Clock*¹⁹ and *Per2*^{*Brdm1*} mutants both have enhanced dopaminergic tone, which may figure prominently in changes in mood-related behaviors [13].

Although light is the dominant time cue for the synchrony of circadian rhythms, non-photic stimuli (feeding, social interactions) also provide circadian cues [17], and recent evidence suggests that exposure to scheduled social cues mitigates symptoms of affective disturbances [10]. Because the robustness of circadian output diminishes with advancing age [18], interactions between clock function, social zeitgebers and affective state in older individuals warrant additional attention.

Here we used a model of chronic circadian arrhythmia generated noninvasively, which circumvents many confounds associated with common models of circadian arrhythmia (brain lesions, bright LL treatments, genetic mutations [19,20]). CRs in sleep/wake, body temperature, hormone secretion and locomotor activity of Siberian hamsters can be

eliminated by light treatments administered just once in adulthood (see *Methods, Section 2.4*). This noninvasive method for permanently eliminating CRs has the advantage of allowing animals to remain genetically and surgically undisturbed in the presence of a normal light:dark cycle. Circadian arrhythmic hamsters were used to investigate interactions between circadian desynchrony and aging on depressive- and anxiety-like affective behavior in young adult and aged animals. We also examined whether a social manipulation (group housing) would attenuate effects of arrhythmia on emotionality.

Lastly, emerging evidence indicates that glucocorticoids and neuroinflammation figure prominently in the generation of emotional behaviors [21-23]. Acute induction of proinflammatory cytokines (e.g., LPS-induced IL-1 β , IL-6, TNF α production) in limbic brain regions (hippocampus, frontal cortex) modulates the cognitive, emotional and behavioral symptoms of acute sickness; in addition, modest, but constitutive and chronic, increases in limbic cytokines are also associated with depressive-like behavior [21,24-26]. One mechanism by which CNS cytokine expression affects emotional behavior is through the induction of indolamine-2,3-deoxygenase (*Ido*) mRNA expression, which metabolizes tryptophan into L-kynurenine [27]. Decreases in tryptophan can reduce synaptic serotonin [28] and induce neuronal damage via excitotoxic mechanisms [29,30] and oxidative stress [31]. Increases in hippocampal and cortical IDO expression parallel the time-course of acute infection-induced sickness behaviors [32,33], and IDO inhibition blocks depressive and anxiogenic effects of acute neuroinflammation [32,34]. Therefore, in the present work we also characterized HPA axis function and limbic expression of *IL-1 β* and *Ido*, to examine whether changes in these pathways are associated with arrhythmia-induced emergence of affective behaviors.

2. Material and Methods

2.1. Animals

Siberian hamsters (*Phodopus sungorus*) were derived from a long-day, 15L:9D photoperiod (LD; lights off at 18:00 h CST) breeding colony maintained at the University of Chicago. Hamsters were housed in polypropylene cages, with food (Harlan, Teklad) and filtered tap water provided *ad libitum*; cotton nesting material was also available in the cage. Ambient temperature and relative humidity were held constant at 19 $\pm$ 2°C and 53 $\pm$ 10%, respectively. All procedures conformed to the USDA Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

2.2. Social housing manipulations

To evaluate whether social housing mitigates effects of circadian disruption on emotional behavior, identical and parallel experiments were performed on socially-isolated and group-housed hamsters. Isolate and Group hamsters were tested on the same days and by the same experimenters. Following behavioral testing, Group hamsters were briefly (<60 min) housed in a neutral cage. For the social investigation test (see below) Group hamsters remained housed together in a new cage, while each cage member was tested individually in the home

cage. After behavioral tests on all residents of a given Group cage were completed, hamsters were returned to the Group cage in unison.

2.3. Aging manipulation

Male offspring were weaned at 18-21 days of age and housed 1-4/cage in the LD breeding colony room. One month prior to circadian rhythm disruption (*DPS procedure*, see below), young and aged hamsters were either single-housed (1/cage; “Isolate”; n=72; 36 young, 36 aged), or remained group housed (3-5/cage; “Group”; n=94; 53 young, 41 aged). Group housed hamsters remained group housed at all times except for: (1) brief intervals during behavioral testing, and (2) an interval of circadian behavioral phenotyping (see *Locomotor activity monitoring*, below), during which Group hamsters were housed 1/cage for 10 days of activity recording, after which they were returned to the Group cage. In all hamsters, the DPS procedure was performed at 4-8 months of age. Affective behavioral testing began 1-2 month after DPS in young hamsters (age at testing = 5-9 months) and 10 months after DPS in aged hamsters (age at testing = 14-18 months).

2.4. Disruptive phase shift (DPS) procedure

Hamsters were subjected to a disruptive phase shift (DPS) procedure to induce circadian arrhythmia. The DPS manipulation destabilizes the hamster circadian pacemaker via treatment with phase-resetting light stimuli, rendering a substantial proportion of hamsters permanently circadian arrhythmic (“ARR”; [35]). Isolate (n=47) and Group (n=67) hamsters were housed for 4 weeks in a 16L:8D photoperiod, then on a single night, a 2 h light pulse was administered during the 5th through 7th h of the dark phase. On the next day, the 16L:8D photocycle was phase-delayed by 3 h, via extension of the light phase (new lights off: 21:00 h). The DPS protocol typically renders approximately 50% of hamsters permanently circadian arrhythmic (“ARR”; Ruby et al., 2004). Control hamsters (Isolate: n=25; Group: n=27) were subjected to the 3 h phase-delay but did not receive the 2 h light pulse on the preceding night (sham-DPS).

2.5. Locomotor activity monitoring

Following the DPS treatment, home-cage locomotor activity was assessed using passive infrared motion detectors (Coral Plus, Visonic, Bloomfield, CT) positioned outside the cage (22 cm above the cage floor). Motion detectors registered activity when 3 of 27 zones were crossed. Activity triggered closure of an electronic relay recorded by a computer running ClockLab software (Actimetrics, Evanston, IL). Cumulative activity counts were collected at 6 min intervals. Activity data were collected in 10-day blocks, 2-3 months after DPS was administered (cf. Ruby et al., 2004). In order to determine their circadian phenotype, Group housed hamsters were temporarily socially-isolated during the interval locomotor activity monitoring, and were returned to their original social cages (with cagemates) following 10 days of activity data collection.

2.6. Circadian waveform analyses

The presence/absence of CRs was determined using criteria identical to those described in prior reports of DPS-induced arrhythmia in this species, and χ^2 periodogram analyses

(ClockLab; Actimetrics) were performed on 10-day blocks of activity data, 2-3 months after the phase-shift was administered (cf. [20,35]). Peaks in the χ^2 periodogram were considered statistically significant if they exceeded the 99.9% confidence interval limit ($P < 0.001$). Hamsters were considered arrhythmic (ARR) if there were no significant peaks in the periodogram in the circadian range, activity was distributed throughout the light-dark cycle, and daily activity onsets and offsets could not be identified via visual inspection of the periodogram. Hamsters with significant circadian activity in the χ^2 periodogram were considered entrained (ENTR) [20,35].

2.7. Emotional behavior testing

Testing of depressive- and anxiety-like behaviors commenced 4-8 weeks after circadian behavioral phenotyping was completed. A Porsolt forced swim test (PFST) and a social investigation (SI) test were used to assess depressive-like behaviors; an open field test (OFT) and a light-dark shuttlebox (LDS) test were used to assess anxiety-like behaviors. In addition, a limb strength test was performed (tests described below). Tests were conducted during a 2 h window centered at the midpoint of the light phase. Testing was performed in the following order: PFST, SI, LS, OFT, LDS; each behavior test was separated in time by 1 week.

In the majority of these behavioral tests, hamsters were removed from the home cage for brief intervals of time. Handling and removal from the home cage are inherently stressful procedures. Such stress may occur in addition to the stressful attributes of several of the behavioral paradigms (e.g., the PFST and the OFT). The extent to which age and circadian phenotype interact with any effect of handling stress on behavioral performance is not presently known. Stress associated with removal from the home cage may contribute to variance in behavioral responses reported here.

The experimental design necessitated performing the affective behavioral tests in a fixed order. We cannot exclude the hypothesis that exposure to initial behavioral tests (e.g., the Porsolt test) influenced performance on subsequent tests, nor can we exclude effects of repeated handling on behavioral performance. We note, however, that the tests were performed in identical order in for all treatment groups.

2.7.1. Porsolt forced swim test—This standard measure of depressive-like behavior operationalizes behavioral despair as the time spent floating (versus active escape) in an inescapable water tank (27 °C, 14 cm diameter $\times$ 10 cm depth), which can be reversed by antidepressant treatment [36]. On the first day of testing, hamsters were exposed to the water tank for 15-min (pre-test). On day 2, a 5-min test was performed. The amount of time spent swimming and climbing (escape behaviors), and floating (behavioral despair) were measured at 5 second intervals using a real-time sampling technique [24,37].

2.7.2. Social investigation test—SI is a validated procedure for quantifying the intensity of social motivation; it is a sensitive measure for social withdrawal during the acute response to simulated bacterial infection [38,39]. Social exploration was measured by presenting a novel juvenile (prepubertal, <25 days of age) to a focal experimental hamster animals in the focal animal's home cage [39]. The test lasted 5 min, and the duration of time

spent by the focal hamster engaged in social investigation of the juvenile (anogenital sniffing, grooming, and body sniffing) during this interval was recorded [38]. Interactions were scored by an observer who was unaware of the experimental treatment conditions or the experimental hypothesis. Immediately prior to the SI test, Group hamsters were transferred collectively into a neutral cage and were then tested individually (with the stimulus animal) in the original home cage.

2.7.3. Limb strength—A Plexiglas hamster habitat tube (Toob-A-Trail, Penn-Plax, Inc., Garden City, NY, USA) was suspended vertically, using a ring stand and clamps, approximately 30 cm above a clean hamster cage with one open end of the tube facing downward. A hamster was inserted into the lower end of the tube and held in place until it gripped the inner contours of the tube. The hamster was then released, and the time until the hamster fell was recorded. The cage into which the hamster fell contained bedding (to a depth of 5 cm) in order to cushion the fall from the tube. This test was repeated 3 times in succession with 1 min rest breaks in between trials. The mean hanging time over 3 trials served as a measure of limb strength [40].

2.7.4. Open-field behavior—This test measures exploratory anxiety by quantifying activity in the periphery and in the center of a novel open arena (1.3 m²). Decreases in center entries and time spent in the center region are interpreted as anxiety-like behaviors [41]. Hamsters were placed in a randomly selected corner of the Plexiglas arena, and locomotor activity behavior was videotaped for 10 min. Scoring of locomotor activity in the OFT was performed by an experimenter on automated system (Ethovision, Noldus Information Technology, Leesburg, VA USA) which determined the total distance travelled, the latency to enter the center of the arena, the number of entries into the arena center, and the total duration of time spent in the arena center.

2.7.5. Light-dark shuttlebox test—A light-dark shuttlebox apparatus (0.27 m long × 0.27 m wide, 0.21 m high) constructed of Plexiglas with a wire floor was used as an additional assay of anxiety-like behavior. One half of the total area of the box (0.27 m wide × 0.135 m long) was completely darkened using an opaque Plexiglas chamber insert. Hamsters were placed in the darkened chamber, and behavior was videotaped for 10 min. Scoring of locomotor activity in the open (illuminated) portion of the arena was performed by an experimenter who was blind to the experimental treatments and hypotheses. The latency to first enter the illuminated portion of the apparatus, the number of transitions between the two compartments, and the total amount of time spent in the dark compartment were scored as indices of anxiety-like behavior.

2.8. Baseline and stress-induced cortisol

>2 weeks after behavioral testing was completed, HPA axis reactivity was assessed. Baseline cortisol concentrations were measured at the midpoint of the light phase in both aged and young hamsters expressing ENTR and ARR circadian phenotypes. In addition, cortisol responses to restraint stress were quantified in aged hamsters. To elicit stress responses, hamsters were placed in restraint stress tubes (length: 10 cm; diameter: 4.5 cm; [42]) at the midpoint of the light phase. Hamsters remained in restraint for 2 h. Control (non-

stressed) hamsters were allowed to remain undisturbed in the home cage. At the conclusion of the 2 h interval, blood (150 μ l) was obtained via the retroorbital sinus under isoflurane anesthesia. Stress and control procedures were performed in rooms that were acoustically isolated from one another. Blood collections were performed in a pseudo-random order, also in separate room. Following the procedures, hamsters remained separated from the general housing room until all blood collections for the day were completed. Animal handling during the blood collection was kept to a minimum (<1 min). Blood samples were kept on ice for <1 h and were centrifuged at $300 \times g$ for 30 min at 4°C. Plasma was extracted and stored at -70°C until assayed for cortisol.

2.9. Brain tissue collection

Following measurements of HPA axis reactivity, hamsters were euthanized by rapid decapitation at the midpoint of the light phase. Only brains from control (non-stressed) hamsters were used for this analysis. Whole hypothalamus and frontal cortex were rapidly dissected, frozen on dry ice, and transferred to -80°C until RNA was extracted. The hippocampus was dissected after a 24 h soak in RNAlater (Qiagen) and subsequently frozen at -80°C.

2.10. Quantitative real-time PCR

RNA was extracted from brain tissue using RNeasy (Qiagen, PLUS Mini-Kit). Nucleic acid concentration and quality were determined by spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE). cDNA was synthesized using Superscript III (Invitrogen, Carlsbad, CA) and stored at -20°C until quantitative PCR was performed.

Primers for interleukin-1 β (*IL-1 β*) and indolamine-2,3-deoxygenase (*Ido*) were designed based on conserved regions of mouse, rat and human sequences using PrimerExpress and optimized for use in Siberian hamsters (Table S1). PCR sequences were confirmed at the University of Chicago DNA sequencing facility, and a standard nucleotide-nucleotide BLAST was used to determine sequence specificity.

qPCR reactions were performed using 2 μ l of cDNA in a BioRad CFX384 system (Hercules, CA, USA). Following initial denaturation at 95°C for 30 s, we performed 39 cycles of: (1) 95°C for 10 s, (2) annealing at temperatures that varied based on the target mRNA (see Table S2) for 30 s, and (3) extension at 72°C for 30 s. Identification of mRNA levels was accomplished with iQ Sybr Green Supermix (BioRad). After the final PCR cycle, all reactions were subjected to melting curve analyses to determine the quality and specificity of each reaction. Select amplicon sequences were run in a 2.5% agarose gel to confirm PCR product size. Reaction efficiencies (E) and cycle thresholds (CTs) were calculated using PCR Miner [43]. Average E value across all reactions approximated 1.0 (Mean=1.08; SD=0.10). Quantification of mRNA expression of each target gene was determined using $2^{-(\Delta\Delta Ct)}$ calculation and is reported relative to expression of the reference mRNA, glyceraldehyde phosphate dehydrogenase (GAPDH).

2.11. Serum cortisol concentrations

Cortisol was measured according to the manufacturer's instructions from 100 μ L duplicate plasma samples (diluted 1:40) using an EIA (Correlate-EIA; Enzo Life Sciences) that has been validated for this species [44]. The cortisol EIA has a sensitivity of <57 pg/mL, an intra-assay coefficient of variation (CV) of <10.5%, and an interassay CV of <8.6%.

2.12. Effects of affective state on circadian response to DPS

A separate experiment examined the hypothesis that an innate, pre-existing depressive-like state may predispose hamsters towards susceptibility to DPS-induced arrhythmia. Thus, in a separate cohort of unmanipulated, single-housed male hamsters (n=66), depressive-like behaviors were measured via the PFST at 3-4 months of age, and 4 weeks later, all hamsters were exposed to the DPS treatment. Locomotor activity monitoring and circadian waveform analyses were performed 2-3 months after the DPS treatment in a manner identical to that described above (see *Locomotor activity monitoring*, *Circadian waveform analyses*).

2.13. Statistical analyses

Chi-square tests were used to evaluate the significance of the distribution of circadian phenotypes across experimental groups. Effects of age, circadian phenotype and social housing condition on dependent variables were assessed using ANOVA (Statview 5.0; SAS Institute, Cary, NC, USA) followed by two-tailed paired t-tests or Fisher's PLSD tests, where appropriate. Pairwise comparisons were only performed if a significant omnibus F statistic was obtained. *A priori* planned comparisons on the effects of social housing manipulations on emotional behaviors were performed without correction for familywise error. For the purposes of clarity in describing the data, effects of age and circadian phenotype on emotional behaviors are discussed separately from effects of social manipulations; however, all ANOVA statistics reported are derived from the complete 2 (age) $\times$ 2 (circadian phenotype) $\times$ 2 (social condition) factorial ANOVA. Differences were considered significant if $P \leq 0.05$.

3. Results

3.1. DPS treatment caused circadian arrhythmia

Overall, DPS treatment induced circadian arrhythmia ("ARR") in 46% (26/57) of young hamsters, and in 44% (25/57) of aged hamsters ($\chi^2=0.04$, $P>0.80$). A minority of hamsters (8 young, 2 aged) subjected to DPS exhibited free-running locomotor activity which did not entrain to the L:D cycle; these hamsters were omitted from subsequent study [45]. Social environment did not affect the circadian response to DPS. Among young hamsters, DPS caused arrhythmia in 35% of Isolate- and in 51% of Group-housed ($\chi^2=0.06$, $P>0.90$); among aged hamsters, DPS triggered 48% of Isolate- and 40% of Group-housed hamsters to become ARR ($\chi^2=0.61$, $P>0.40$). Representative locomotor activity records appear in Figure S1.

In contrast to DPS, sham-DPS treatment resulted in behavioral arrhythmia in 0 of 32 young hamsters ($\chi^2=20.6$, $P<0.001$; vs. young DPS group) and in only 3 of 20 of aged hamsters ($\chi^2=5.33$, $P<0.05$; vs. aged DPS group). A total of 50 (n=21 young, n=29 aged) hamsters

retained entrained locomotor activity patterns following the DPS treatment. CR amplitude did not differ between entrained hamsters that received DPS and those that received sham-DPS (young: $F_{1,51}=0.24$, $P>0.60$; aged: $F_{1,44}=0.77$, $P>0.30$); thus, to increase statistical power, these hamsters were combined into a single circadian entrained (ENTR) treatment group within each age cohort (young-Isolate: $n=21$, young-Group: $n=32$; aged-Isolate: $n=19$, aged-Group: $n=27$).

3.2. Circadian arrhythmia induced depressive-like behavior

In the forced swim test, ARR hamsters floated significantly longer than ENTR hamsters ($F_{1,140}=4.00$, $P<0.05$; Fig. 1A). Effects of circadian arrhythmia on this measure were only evident in aged hamsters, however, which exhibited total floating durations that were 5-fold greater than those of age-matched ENTR hamsters ($P<0.001$). In contrast, among young hamsters total float time did not differ between ENTR and ARR groups ($P>0.40$; Fig. 1A). Among ENTR hamsters, floating time did not differ between young and aged groups ($P>0.60$). Similar effects were evident in the latency to the first floating bout ($F_{1,140}=6.88$, $P<0.01$; Fig. 1B), although the effect of arrhythmia on the number of floating bouts was not significant ($F_{1,140}=1.54$, $P>0.20$; Fig. 1C). Limb strength was unaffected by age ($F_{1,145}=0.19$, $P>0.60$) or circadian phenotype ($F_{1,145}=0.59$, $P>0.40$; Fig. 1D).

In the social investigation test, circadian phenotype and age interacted to affect social motivation ($F_{1,142}=5.54$, $P<0.05$; Fig. 2). Aged-ENTR hamsters exhibited significantly more social investigation relative to young-ENTR hamsters ($P<0.001$), whereas an effect of age was not evident in ARR hamsters ($P>0.40$). Relative to ENTR controls, aged-ARR hamsters exhibited less social investigation ($P<0.05$), and young-ARR hamsters exhibited significantly more social investigation ($P<0.05$; Fig. 2).

3.3. Circadian arrhythmia decreased anxiety-like behavior

Neither age nor circadian phenotype affected total locomotor activity in the open field apparatus (age: $F_{1,139}=0.63$, $P>0.40$; circadian phenotype: $F_{1,139}=0.09$, $P>0.70$; Fig. 3A). However, age and circadian phenotype interacted to affect the amount of time spent exploring the center of the arena ($F_{1,139}=8.44$, $P<0.005$; Fig. 3B). Effects of arrhythmia on center exploration time were apparent only in aged hamsters (aged: $P=0.001$; young: $P>0.15$). Age and arrhythmia also interacted to affect the number of entries into the arena center ($F_{1,139}=6.64$, $P=0.01$; Fig. 3C). Relative to aged-ENTR controls, aged-ARR hamsters tended to exhibit a greater number of center entries ($P=0.07$); an opposite effect of arrhythmia was observed in young animals, with young-ARR hamsters exhibiting fewer center entries relative to controls ($P<0.05$; Fig. 3C). Among ENTR animals, aged animals exhibited significantly fewer center entries relative to young animals ($P=0.01$). Lastly, age and circadian phenotype also interacted to affect the latency to enter the arena center ($F_{1,139}=4.10$, $P<0.05$; Fig. 3D). Young-ARR hamsters were significantly slower to enter the arena center relative to young-ENTR and aged-ARR hamsters ($P=0.05$, both comparisons).

In the light-dark shuttlebox test, aged hamsters spent more total time in the illuminated region of the apparatus ($F_{1,138}=7.94$, $P<0.01$; Fig. 4A), and age and circadian phenotype interacted to affect time in the illuminated section ($F_{1,138}=5.83$, $P<0.05$). Aged-ARR

hamsters spent significantly more time in the illuminated region relative to aged-ENTR hamsters ($P < 0.05$); in contrast, among young hamsters, arrhythmia did not induce a significant change in this measure of anxiety-like behavior ($P > 0.05$). Age and circadian phenotype were without effect on the latency to first enter the illuminated chamber (age: $F_{1,138} = 2.58$, $P > 0.10$; circadian phenotype: $F_{1,138} = 0.16$, $P > 0.60$; Fig. 4B), or the number of transitions between light and dark chambers (age: $F_{1,138} = 0.09$, $P > 0.70$; circadian phenotype: $F_{1,138} = 0.10$, $P > 0.70$; Fig. 4C).

3.4. Social housing mitigated the effects of arrhythmia on select emotional behaviors

Among aged hamsters, social housing eliminated the effect of circadian arrhythmia on depressive-like behavior in the PFST (social condition $\times$ circadian phenotype interactions: total floating: $F_{1,70} = 6.29$, $P = 0.01$; latency to first float: $F_{1,70} = 4.2$, $P < 0.05$; number of floating bouts: $F_{1,70} = 3.56$, $P = 0.06$; Fig. 1, right panels). In young hamsters, however, depressive-like behaviors were not affected by social housing (social condition main effects and social condition $\times$ circadian phenotype interactions: $F_{1,70} < 2.09$, $P > 0.15$, all comparisons; Fig. 1, right panels).

In the social investigation test, social housing markedly diminished social investigation behavior ($F_{1,142} = 148.4$, $P < 0.001$). Social housing also eliminated the effects of circadian arrhythmia on social motivation; this effect was significant in both aged (social condition $\times$ circadian phenotype: $F_{1,67} = 3.99$, $P < 0.05$) and young (social condition $\times$ circadian phenotype: $F_{1,75} = 5.01$, $P < 0.05$) hamsters (Fig. 2, right panels).

Anxiety-like behaviors in the open field test were also altered by the social housing manipulation (Fig. 3, right panels). Group-housed hamsters spent significantly less time in the center of the arena ($F_{1,139} = 4.47$, $P < 0.05$), an effect which was largely due to a reduction in center exploration time in group-housed young, entrained hamsters relative to their isolate controls ($P < 0.05$). Social condition did not affect the number of center entries ($F_{1,139} = 0.10$, $P > 0.70$) or the latency to enter the arena center ($F_{1,139} = 0.03$, $P > 0.80$; Fig. 3, right panels). Effects of circadian arrhythmia on center time and center entries were absent in group-housed hamsters (center time: $F_{1,81} = 0.25$, $P > 0.60$; center entries: $F_{1,81} = 0.18$, $P > 0.60$).

Main effects of social housing were not evident on measures of anxiety in the LD shuttlebox test (total time in illuminated chamber: $F_{1,138} = 2.16$, $P > 0.10$; latency to enter illuminated chamber: $F_{1,138} = 1.07$, $P > 0.30$; number of transitions: $F_{1,138} = 0.32$, $P > 0.50$; Fig. 4, right panels). Among group-housed hamsters, circadian phenotype significantly affected the amount of time spent in the light region of the chamber ($F_{1,80} = 13.5$, $P < 0.001$): both young ($P < 0.01$) and aged ($P < 0.05$) ARR hamsters spent more time in the illuminated region relative to their respective group-housed ENTR controls. Group-housed aged hamsters were faster to enter the illuminated region relative to group-housed young hamsters ($F_{1,80} = 9.63$, $P < 0.005$), irrespective of circadian phenotype ($F_{1,80} = 0.33$, $P > 0.50$).

Baseline cortisol concentrations were not affected by the social housing manipulation ($F_{1,69} = 1.81$, $P > 0.10$), and social housing did not significantly affect the stress-induced increase in cortisol ($F_{1,41} = 1.87$, $P > 0.10$; Fig. 5, right panels). Among group-housed entrained hamsters, restraint stress elicited significant increases in cortisol ($P < 0.05$), but this

effect was not evident in ARR hamsters ($P>0.40$), likely due to a high variance and a very small sample size ($n=3$) in baseline measures for this group.

3.5. Circadian arrhythmia did not affect H PA axis activity

Overall, baseline plasma cortisol concentrations were higher in aged relative to young hamsters ($F_{1,69}=7.01$, $P<0.05$), but were not affected by circadian phenotype ($F_{1,69}=0.44$, $P>0.50$; Fig. 5A). Among aged hamsters, restraint stress provoked a marked increase in plasma cortisol concentrations ($F_{1,41}=19.2$, $P<0.001$), but the magnitude of the increase in plasma cortisol was unaffected by circadian phenotype ($F_{1,41}<0.01$, $P>0.90$; Fig. 5B).

3.6. Hippocampal and frontal cortical IL-1 β mRNA expression

Age ($F_{1,59}=7.17$, $P<0.01$) and social housing ($F_{1,59}=17.4$, $P<0.0001$), and an interaction thereof ($F_{1,59}=7.26$, $P<0.01$), affected hippocampal IL-1 β mRNA expression (Fig. 6A). IL-1 β mRNA was significantly greater in socially-isolated relative to group housed hamsters, although this effect was restricted to young animals (Fig. 6A). Circadian arrhythmia increased hippocampal IL-1 β expression in young, socially-isolated hamsters ($P<0.01$), but not in any other group ($P>0.60$, all comparisons).

IL-1 β expression in frontal cortex was not affected by age ($F_{1,56}=0.14$, $P>0.70$), social housing condition ($F_{1,56}=2.01$, $P>0.10$) or circadian phenotype ($F_{1,56}<0.01$, $P>0.90$; Fig. 6B).

3.7. Hippocampal and frontal cortical Ido mRNA expression

Social housing markedly affected hippocampal Ido mRNA expression ($F_{1,59}=20.6$, $P<0.0001$; Fig. 6C); hippocampal Ido expression was significantly greater in socially-isolated than in group-housed hamsters. Although main effects of age ($F_{1,59}=0.67$, $P>0.40$) and circadian phenotype ($F_{1,59}=2.27$, $P>0.10$) were not significant, age and social housing ($F_{1,59}=3.52$, $P=0.066$) tended to affect Ido expression: in young hamsters of both circadian phenotypes, hippocampal Ido did not differ between isolates and group-housed groups (ENTR: $P>0.05$; ARR: $P>0.10$), whereas in aged hamsters, Ido was markedly higher in ENTR-isolate than in ENTR-group hamsters ($P<0.0005$), but isolated and group-housed ARR hamsters did not differ significantly ($P>0.10$). Age and circadian phenotype also tended to affect hippocampal Ido expression ($F_{1,59}=3.77$, $P=0.057$), with trends towards increased Ido expression in ENTR relative to ARR young hamsters ($P=0.06$, both housing conditions), but not in aged hamsters ($P>0.20$, both housing conditions).

Age ($F_{1,58}=0.71$, $P>0.40$), circadian phenotype ($F_{1,58}=0.01$, $P>0.90$), and social housing condition ($F_{1,58}=0.32$, $P>0.50$) were without effect on frontal cortical Ido mRNA expression (Fig. 6D). Interactions among these variables were likewise not significant ($P>0.40$, all comparisons).

3.8. Depressive-like behavior did not predict the circadian response to DPS treatment

Among the 66 hamsters subjected to the PFST prior to DPS treatment in Experiment 2, 27 (41%) remained ENTR, 31 (47%) became ARR, and 8 (12%) exhibited free running rhythms. Depressive-like behavior exhibited in the PFST prior to the DPS treatment did not

differ between hamsters that later exhibited ARR and ENTR responses to DPS (Fig. 7). Total duration of floating behavior ($F_{1,56}=2.71$, $P>0.10$; Fig. 7A), latency to the first floating bout ($F_{1,56}=0.32$, $P>0.50$; Fig. 7B), and the total number of floating bouts ($F_{1,56}<0.01$, $P>0.90$; Fig. 7C) were comparable among ARR and ENTR phenotypes. The incidence of DPS-induced ARR did not differ between hamsters in the highest and lowest quartiles for floating behavior in the PFST: 11 of 15 hamsters highest in floating behavior (75-100%ile), and 9 of 15 hamsters lowest in floating behavior (0-25%ile), became ARR following DPS ($\chi^2=0.60$, $P>0.40$).

3.9. Circadian arrhythmia did not affect reproductive, immunological, or endocrine tissue masse

Circadian arrhythmia and social housing manipulations did not affect testis (circadian phenotype: $F_{1,136}=3.03$, $P>0.05$; social housing: $F_{1,136}=2.51$, $P>0.10$; Fig. S2, panel A), spleen (circadian phenotype: $F_{1,136}=0.40$, $P>0.50$; social housing: $F_{1,136}=0.03$, $P>0.80$; Fig. S2, panel B), or paired adrenal mass (circadian phenotype: $F_{1,136}=0.84$, $P>0.30$; social housing: $F_{1,136}=0.42$, $P>0.50$; Fig. S2, panel C).

4. Discussion

Aged hamsters lacking a functional circadian system exhibited increases in depressive-like behavior accompanied by decreases in anxiety-like behavior. In younger hamsters, emotional effects of circadian arrhythmia manifested in a less consistent manner: young arrhythmic (ARR) hamsters showed greater levels of social motivation than age-matched entrained (ENTR) hamsters, but also greater levels of anxiety-like behavior in the OFT. Social housing abolished effects of arrhythmia on despair and social withdrawal, and on OFT measures of anxiety, but anxiolytic effects of arrhythmia in the LD test persisted in aged animals. HPA axis function was not associated with emotional behaviors: resting and stress-induced plasma cortisol concentrations were comparable in ENTR and ARR hamsters. Moreover, circadian arrhythmia alone was without effect on hippocampal or cortical *IL-1 β* and *Ido* mRNA expression in aged hamsters, but social housing significantly decreased hippocampal *IL-1 β* and *Ido* mRNAs. Together, the data identify novel emotional effects of circadian dysfunction in aged animals and its amelioration by social housing. Clear associations between arrhythmia and either HPA axis function or constitutive limbic proinflammatory activity were not evident, but the present data suggest that social housing markedly attenuates hippocampal *IL-1 β* and *Ido* activity, which may contribute to the ameliorating effects of social housing on emotional behaviors.

One obstacle to addressing the functional significance of the circadian system in the regulation of affective state has been the lack of an unconfounded model system. SCN lesions reliably eliminate CRs, but also damage substantial adjacent hypothalamic tissue, and cause nonspecific production of stress hormones [46,47], along with glial scars, inhibition of tuberoinfundibular dopamine signaling and hyperprolactinemia [46]; in addition, CNS inflammatory responses persist for long intervals after surgery [48,49]. Perhaps not surprisingly, effects of SCN lesions on emotional behaviors are variable, ranging from null to anti-depressive [50-52]. Prolonged exposure to constant light (LL) gradually induces circadian arrhythmia, and LL induces depressive-like behavior and

reduces anxiety-like behavior in mice [53]. Select clock gene mutations eliminate circadian rhythms [1] and trigger idiosyncratic changes in emotional behaviors, but clock genes are present in all tissues and are pleiotropic in function [54-56]. Pleiotropy is relevant towards interpreting the diverse changes in emotional behaviors evident in clock gene knockout models: affective changes may be due to the loss of circadian time information, *per se*, in the CNS, but also may be a consequence of the deletion of genes that play key roles in cellular metabolic and neuroendocrine pathways [57-59]. Abnormal metabolic activity is commonly associated with mood disorders and their treatments, and it remains plausible that mood alterations in common mutant mouse models are due in part to pathologies that emerge in peripheral metabolic processes [13]. The present work indicates that arrhythmia alone, absent lesions, LL or genetic mutations, is sufficient to alter emotional behaviors, providing novel convergent evidence in support of the importance of circadian timing information in the regulation of emotional state.

Social exposure ameliorated effects of circadian disruption on emotional behavior. Single-housed, aged-ARR hamsters exhibited more floating behavior in the PFST, less social investigation, and spent more time in the center of the OFT arena and in the light chamber of the LD test. With the exception of the LD test, each of these effects was absent in group-housed hamsters, suggesting that some aspect(s) of the social environment buffers hamsters from most arrhythmia-induced emotional dysregulation. Light is a potent zeitgeber for the circadian system, but circadian clocks are also sensitive to non-photoc time cues; particularly effective in this regard are social cues [17]. Socially-derived non-photoc time cues may also ameliorate affective disturbances in humans: in patients with bipolar I disorder, interpersonal and social rhythms therapy (IPSRT) delays the onset of affective episodes, and IPSRT patients are less likely to exhibit episode recurrence, an effect that is mediated by increased regularity of social routines [10,60]. IPSRT is a modified form of interpersonal psychotherapy based on a 'social Zeitgeber' hypothesis [61]: that irregular daily routines in afflicted (untreated) individuals destabilize biological circadian rhythms, which give rise to, or exacerbate, affective episodes. One emphasis of IPSRT is the maintenance of regularly-timed daily social and individual activities [62]. Although the present experimental approach did not deliver daily 'pulses' of social interaction, the data nevertheless indicate that increased conspecific social contact mitigates the effects of circadian disruption on depressive- and anxious-like behaviors.

The present work characterizes changes in several aspects of constitutive limbic neuroinflammatory activity associated with aging, social exposure, and circadian arrhythmia. Arrhythmia alone was sufficient to elevate hippocampal *IL-1 β* expression in young hamsters, although this was not associated with any changes in emotional behavior. Group housing, however, was associated with decreases in constitutive expression of *IL-1 β* and *Ido* in the hippocampus, which paralleled the elimination of ARR-induced depressive-like and anxiolytic-like behaviors in older hamsters. The data indicate that social exposure alone, absent any precipitating immune challenge (e.g., LPS) is sufficient to suppress constitutive proinflammatory cytokine and serotonin-depleting enzyme expression in limbic brain regions.

Advanced age gradually impairs immune function [22]. One consequence of the decreases in T cell function and anti-inflammatory endocrine activity [63] in aged animals is a heightened CNS proinflammatory tone [64]. In whole-brain assays, aged mice exhibit elevated constitutive and LPS-induced neuroinflammatory gene expression [65]. The incidence of circadian disruptions also increases markedly with advanced age [18,66-69], but interactions between aging and clock function in the regulation of emotional state have received only limited investigation [70]. The present data indicate that older hamsters may be more susceptible to emotional effects of arrhythmia, but unlike in mice, constitutive *IL-1 β* expression was not elevated in aged relative to young hamsters. Hamsters may simply differ from mice in terms of aging-related increases in proinflammatory cytokine expression; alternatively, immunosenescence-associated increases in brain *IL-1 β* may be specific to select brain regions: aging markedly increased constitutive *IL-1 β* expression in a whole brain assay [65], however, similar effects were not evident when analyses were restricted to hippocampus or cerebellum [71].

Aged hamsters that became ARR following the DPS treatment exhibited changes in emotional behavior. One interpretation of this outcome is that circadian arrhythmia dysregulates emotional behavior. An alternative hypothesis, however, contends that aged hamsters with dysregulated emotional behavior (depressive-like and anxiolytic-like) were merely more susceptible to the arrhythmia-inducing effects of the DPS treatment. Indeed, for reasons that remain unknown, the DPS treatment does not induce circadian arrhythmia in all hamsters [35,72]. Therefore, in *Experiment 2*, we evaluated the alternative hypothesis by examining one aspect of emotional behavior (behavioral despair) prior to delivering the DPS treatment. Consistent with prior reports, almost half (47%) of DPS-treated hamsters became ARR, but behavior exhibited in the PFST prior to DPS was comparable in hamsters that later exhibited ARR and ENTR circadian phenotypes. This outcome is consistent with the conjecture that arrhythmia gives rise to changes in emotional behavior, and not vice versa.

The present data bear a striking similarity to recent work documenting increased depressive-like and decreased anxiety-like behaviors in female Siberian hamsters exposed to dim nocturnal illumination (dLAN; [73]). dLAN increased behavioral despair and anhedonia, and increased exploratory behavior in an elevated plus maze [73], suggesting that low-level nocturnal illumination is sufficient to disrupt emotional behaviors. These behavioral changes resemble the despair and OFT exploratory responses to ARR in the present report. dLAN effects manifest in changes in hippocampal physiology, with reductions in spine density on CA1 apical dendrites following dLAN. dLAN can be titrated so as to eliminate melatonin (MEL) secretion [74], without markedly altering circadian entrainment to the overarching light:dark cycle [75]. MEL secretion is also eliminated in ARR Siberian hamsters [76]. In light of widespread reports of MEL effects on mood [77-79], one mechanism by which ARR and dLAN may affect emotional state may be through reductions in MEL secretion. dLAN has likewise been reported to be depressogenic in C3H/HeNHsd mice [80] and in diurnal grass rats [81].

Increased depressive-like behaviors alongside decreased in anxiety-like behaviors is not commonly reported in rodent models, nor is it unprecedented (e.g., [73,82,83]). Bedrosian et al. [73] conjectured that if enhanced exploratory behavior in hamsters treated with dLAN in

any way resembles novelty-seeking behavior, then dLAN hamsters may be exhibiting a depressive subtype in which risk-taking behavior is co-morbid with despair and anhedonia. A similar interpretation may be applied to the present data: in aged hamsters, ARR may be simultaneously inducing a depressive-like state and increasing novelty-seeking behaviors. Further behavioral testing would be required to evaluate this conjecture, however.

5. Conclusions

In summary, the present report documents changes in depressive- and anxiety-like behavior in aged hamsters following the induction of arrhythmia. Additional research on the reciprocal relations between circadian disruption and psychopathological states is important to a complete understanding of how desynchrony of the internal milieu and altered phase relations between circadian clocks and the external environment influence emotional behavior [84]. The arrhythmic hamster model described here is instructive in that it permits profound disruption of CRs in the absence of genetic or stress (bright LL, lesions) manipulations, and here we validate that depressive-like behavior is not a pre-existing condition in hamsters that express the ARR phenotype in response to DPS. Widespread changes in affective behaviors were not evident in young adult hamsters, indeed, arrhythmia provoked increases in social motivation in young males. In all cases where arrhythmia altered affective behavior, such changes were eliminated by social housing manipulations. Social housing decreased hippocampal expression of *IL-1 β* and *Ido*, elements of a neuroinflammatory cascade that has been implicated in emotional responses to innate immune activation. Such data may provide preclinical insights into mechanisms by which social interactions are effective in the management and treatment of bipolar disorder. Taken together, these experiments used a novel animal model of circadian arrhythmia to identify depressive and anti-anxiety effects of profound circadian disruption, their amelioration by social manipulations, and potential neuroimmune mechanisms by which the social environment may attenuate constitutive neuroinflammatory activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Circadian arrhythmia increased depressive-like behavior in aged hamsters

Arrhythmia decreased anxious-like behaviors

Emotional effects of arrhythmia were largely absent in young adult hamsters

Social (group) housing reduced the effects of arrhythmia on emotionality

Social housing also reduced hippocampal IL-1 β and Ido mRNA expression

Figure 1. Depressive-like behavior in the Porsolt forced swim test

Mean $\pm$ SEM (A) time spent floating, (B) latency to first float bout, (C) number of floating bouts, and (D) limb strength of young adult (5-9 months of age) and aged (14-18 months of age) male Siberian hamsters that were previously rendered circadian arrhythmic (ARR) by noninvasive light treatments (DPS treatment) or remained entrained (ENTR) to the 16L:8D photocycle. *** $P < 0.001$ vs. ENTR value, within age and social grouping. Hamsters were housed 1/cage (Socially-isolated) or 3-5/cage (Group-housed) at all times except during behavioral testing. Sample sizes indicated along abscissa of panel A.

Figure 2. Depressive-like behavior in juvenile social investigation test

Mean $\pm$ SEM time spent engaged in active social exploration of a juvenile by young adult and aged male Siberian hamsters that were previously rendered circadian arrhythmic (ARR) or remained entrained (ENTR). Hamsters were housed 1/cage (Socially-isolated) or 3-5/cage (Group-housed) at all times except during behavioral testing. * $P < 0.05$ vs. ENTR value, within age and social grouping. ### $P < 0.001$ vs. YOUNG value, within circadian phenotype and social grouping. \$\$\$ $P < 0.001$ vs. Socially-isolated value, within age and circadian phenotype grouping. Sample sizes indicated along abscissa of panel A.

Figure 3. Anxiety-like behavior in the open field test

Mean $\pm$ SEM (A) total distance travelled in an open field test arena, (B) total elapsed time spent in the arena center, (C) number of entries into the arena center, and (D) latency to first center entry of young adult and aged male Siberian hamsters that were previously rendered circadian arrhythmic (ARR) or remained entrained (ENTR). Hamsters were housed 1/cage (Socially-isolated) or 3-5/cage (Group-housed) at all times except during behavioral testing. * $P < 0.05$ vs. ENTR value, within age and social grouping. # $P < 0.05$, ## $P < 0.01$ vs. YOUNG value, within circadian phenotype and social grouping. § $P < 0.05$ vs. Socially-isolated value, within age and circadian phenotype grouping. Sample sizes indicated along abscissa of panel A.

Figure 4. Anxiety-like behavior in the light:dark shuttlebox test

Mean $\pm$ SEM (A) total time spent in the illuminated portion of the light:dark shuttlebox testing arena, (B) latency to first enter the illuminated portion of the arena, and (C) total number of entries into the illuminated chamber of young adult and aged male Siberian hamsters that were previously rendered circadian arrhythmic (ARR) or remained entrained (ENTR). Hamsters were housed 1/cage (Socially-isolated) or 3-5/cage (Group-housed) at all times except during behavioral testing. * $P < 0.05$, ** $P < 0.01$ vs. ENTR value, within age and social

grouping. ++ $P < 0.01$, comparison as indicated. Sample sizes indicated along abscissa of panel A.

Figure 5. Baseline and stress-induced glucocorticoid responses

Mean $\pm$ SEM (A) plasma cortisol concentrations of young adult and aged male Siberian hamsters that were previously rendered circadian arrhythmic (ARR) or remained entrained (ENTR). Hamsters were housed 1/cage (Socially-isolated) or 3-5/cage (Group-housed) at all times except during behavioral testing. + $P < 0.05$, comparison as indicated. (B) Mean $\pm$ SEM baseline (open bars) and stress-elicited (2 h restraint; shaded bars) plasma cortisol concentrations of ARR and ENTR aged male Siberian hamsters that were housed socially-isolated or group-housed. * $P < 0.05$, *** $P < 0.001$ vs. baseline value, within circadian phenotype and social grouping. Sample sizes indicated along abscissa of panel A.

Figure 6. Hippocampal and cortical *IL-1β* and *Ido* expression

Mean $\pm$ SEM (A) hippocampal and (B) frontal cortical *IL-1β* mRNA expression, and (C) hippocampal and (D) frontal cortical *Ido* mRNA expression of young adult and aged male Siberian hamsters that were previously rendered circadian arrhythmic (ARR) or remained entrained (ENTR). Hamsters were housed 1/cage (Socially-isolated) or 3-5/cage (Group-housed) at all times except during behavioral testing. ** $P < 0.01$ vs. ENTR value, within age and social grouping. ### $P < 0.001$ vs. YOUNG value, within circadian phenotype and social grouping. §§ $P < 0.01$, §§§ $P < 0.001$ vs. Socially-isolated value, within age and circadian phenotype grouping. Sample sizes indicated along abscissa of panel A.

Figure 7. Effects of affective state on response to DPS treatment

Mean $\pm$ SEM (A) time spent floating, (B) latency to first float bout, and (C) number of floating bouts in the PFST of randomly-selected adult male Siberian hamsters that were later subjected to the DPS procedure. Hamsters were classified as ARR and ENTR based on behavioral profiles exhibited >1 month following DPS treatment. Sample sizes indicated along abscissa of panel A.