**SUPPORTING INFORMATION**

**Melatonin-dependent changes in neurosteroids are associated with increased aggression in a seasonally breeding rodent**

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**MATERIALS AND METHODS**

***Behavioural testing***

For behavioural trials, staged male dyads were created, which were composed of an experimental animal (i.e., resident) and a stimulus animal (i.e., intruder) of approximately the same age and body mass (± 5%) and with different parents from the experimental animal with which they were paired. The intruder was introduced into the resident’s home cage, which had not been changed for 7 d prior to behavioural testing to allow the experimental (resident) animal to establish its territory1-3. All trials were performed under low red light illumination, and intruders had small, shaved patches on their dorsa for the purpose of identification. Each intruder was used no more than twice per testing period (e.g., group of behaviour trials conducted in a single day, which was approximately 2-3 h in duration and consisted of 12-13 trials). Animals used as intruders (*n* = 20) were individually-housed and maintained in LDs prior to behavioural testing and throughout the study. Behavioural interactions were video recorded, and aggressive and non-aggressive social behaviours were scored for each experimental (resident) animal by three trained, unbiased observers using ODLogTM software (Macropod, Eden Prairie, MN, USA).

***Steroid extraction for LC-MS/MS***

Following bead mill homogenization and a 1 hr incubation period at -20˚C, samples were vortexed and centrifuged at 16,100 x *g* for 10 min at room temperature, and 150 µL of the supernatants was collected into 16 x 100 mm glass test tubes. The centrifugation process was repeated after adding 150 µL 100% acetonitrile with 0.1% formic acid to each sample, and 150 µL of the supernatants was transferred to their respective glass test tubes. Samples were then placed in a 35˚C water bath, dried for 45 min using an Evap-O-Rac system (Cole-Parmer, Vernon Hills, IL, USA), and reconstituted in 175 µL 20% methanol with 0.1% formic acid prior to steroid extraction.

For steroid extraction, C18 OMIX tips (Agilent Technologies, Santa Clara, CA, USA) were primed with 200 µL 100% acetonitrile with 0.1% formic acid and conditioned with 200 µL water with 0.1% formic acid. 175 µL of each sample was drawn up and passed through the tips 20 times, tips were washed with 35 µL water with 0.1% formic acid, and samples were eluted with 175 µL 100% acetonitrile with 0.1% formic acid and collected into 2 mL amber vials fitted with 250 µL glass vial inserts (Agilent Technologies, Santa Clara, CA, USA).

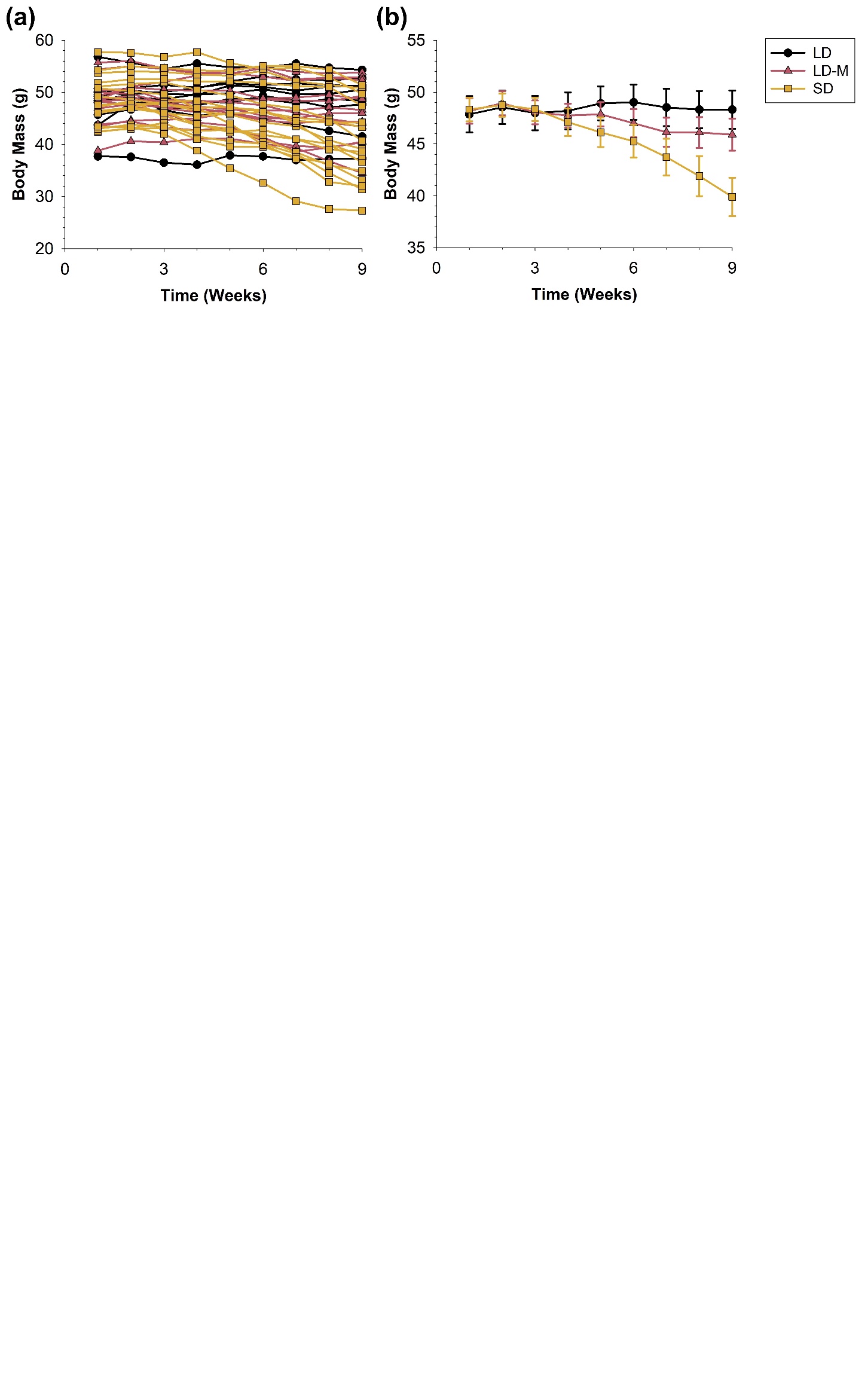
***LC-MS/MS analysis***

For LC-MS/MS analysis, 5 µL of sample was loaded onto an Acclaim PepmapTM C18 RSLC column (75 μm x 25 cm, particle size: 2 μm, pore size: 100 Å; ThermoFisher Scientific, Waltham, WA, USA), and steroids were separated using an acetonitrile-based gradient (solvent A: 0% acetonitrile with 0.1% formic acid, solvent B: 80% acetonitrile with 0.1% formic acid) at a flow rate of 300 nL/min. The column and precolumn were equilibrated with 5 μL of each solvent prior to injection. A 22.5 min gradient was performed as follows: 0 to 0.5 min, 15% to 50% solvent B; 0.5 to 16.5 min, 50% to 100% solvent B; 16.5 to 22.5 min, 100% solvent B. Precursor ions were isolated in scheduled time windows corresponding to their expected retention times, and a window of 1.6 Da was used for isolation prior to fragmentation. Steroids were collisionally fragmented using higher-energy collisional dissociation (HCD) mode with an energy of 35 V, and fragment ions were measured in an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific, Waltham, WA, USA) using a resolution of 120,000 over a mass range of 70-400 Da. For data extraction of fragment ions, a window ± 0.004 Da was used. Prior to quantification, data were processed using a 9 point smooth filter with a baseline window of 102, and each peak was manually inspected to confirm accurate peak detection.

***Statistical analyses***

For multivariate analyses, normality was assessed with Royston’s H tests and homogeneity of covariance matrices was assessed with Box’s M tests using the *assumptions\_manova* function of the *micompr* package4. For univariate analyses, normality was assessed with Shapiro-Wilk tests using the *shapiro.test* function of the *stats* package5, and homogeneity of variances was assessed with Levene’s tests using the *leveneTest* function of the *car* package6. Statistical outliers were examined with Grubbs’ Tests using the *grubbs.test* function of the *outliers* package7. Prior to statistical analysis, circulating progesterone levels were log-transformed to satisfy the assumptions of normality and homogeneity of variances. For multivariate and univariate tests, data were analyzed using the *manova*, *lm,* and *kruskal.test* functions of the *stats* package (one-way MANOVAs, one-way ANOVAs, and Kruskal-Wallis one-way ANOVAs on ranks, respectively)5, the *adonis* function of the *vegan* package (PERMANOVAs)8, the *glht* function of the *multcomp* package (Tukey’s HSD post-hoc tests)9, and the *dunn.test* function of the *dunn.test* package (Dunn’s post-hoc tests)10. In addition, to determine the effects of photoperiodic and melatonin treatment on associations between aggressive behaviour, circulating steroid levels, and neurosteroid levels, Spearman’s rank correlations with a Holm-Bonferroni correction for multiple comparisons were computed for each treatment group using the *corr.test* function of the *psych* package11, and correlation matrices consisting of Spearman’s ρ values were graphed using the *corrplot* function of the *corrplot* package12.

**FIGURES**

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**Figure S1. Male hamsters exposed to short-day photoperiods showed a reduction in body mass.** (a) Individual and (b) mean (± SEM) body mass of long-day animals (LD; black circles), LD animals given timed melatonin injections (LD-M; pink triangles), and animals that were exposed to short-day photoperiods (SD; yellow squares). Body mass was measured weekly for the duration of the study (LD: *n* = 9, LD-M: *n* = 13, SD: *n* = 16).

**TABLES**

**Table S1**. MS/MS transitions for endogenous and isotopically-labeled steroid hormones in Siberian hamster blood and brain tissue.

|  |  |  |  |
| --- | --- | --- | --- |
| **Steroid** | **Retention Time (min)** | **Mass-to-Charge Ratio (m/z)** | |
| **Precursor** | **Fragment** |
| Cortisol | 9.2 | 363.22 | 121.065 |
| Cortisol-d4 | 9.2 | 367.24 | 121.065 |
| Oestradiol | 12.6 | 255.17 | 159.080 |
| Oestradiol-d4 | 12.6 | 259.20 | 161.095 |
| Testosterone | 12.9 | 289.22 | 97.065, 109.065 |
| Testosterone-c3 | 12.9 | 292.23 | 100.075, 112.075 |
| Dehydroepiandrosterone | 13.7 | 271.21 | 253.195 |
| Dehydroepiandrosterone-d6 | 13.7 | 277.24 | 259.230 |
| Progesterone | 17.3 | 315.23 | 97.065, 109.065 |
| Progesterone-d9 | 17.3 | 324.29 | 100.084, 113.090 |

Retention times and mass-to-charge ratios of precursor and fragment ions are shown for each endogenous and isotopically-labeled steroid hormone of interest. Steroids were fragmented using high collision energy dissociation (HCD) fragmentation, and fragment ions were measured using an Easy NanoLC 1200 HPLC coupled to an Orbitrap Fusion Lumos mass spectrometer (see Section 2.8 of Materials and Methods).

**Table S2.** Percent recoveries of isotopically-labeled steroid hormones in Siberian hamster blood and brain tissue following steroid extraction.

|  |  |  |  |
| --- | --- | --- | --- |
| **Isotopic Steroid** | **Blank**  % Recovery | **Blood (3 µL)**  % Recovery | **Brain (0.98-4.41 mg)**  % Recovery |
| Cortisol-d4 | 100 | 72.2 ± 8.93 | 64.2 ± 10.4 |
| Oestradiol-d4 | 100 | 82.6 ± 11.3 | 86.2 ± 9.90 |
| Testosterone-c3 | 100 | 105 ± 9.47 | 102 ± 6.68 |
| Dehydroepiandrosterone-d6 | 100 | 104 ± 9.04 | 103 ± 5.00 |
| Progesterone-d9 | 100 | 117 ± 9.86 | 110 ± 6.05 |

Percent recoveries are presented as mean ± SEM (blood: *n* = 12, brain: *n* = 12) and were calculated relative to the blank sample, which contained 200 µL 100% acetonitrile with 0.1% formic acid spiked with 100 pg cortisol-d4, 200 pg oestradiol-d4, 100 pg testosterone-c3, 100 pg dehydroepiandrosterone-d6, 100 pg progesterone-d9, and 5 ng pregnanediol. The blank sample was processed alongside blood and brain samples during steroid extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

**Table S3.** Accuracy and precision measurements of isotopically-labeled steroid hormones in Siberian hamster blood and brain tissue for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| **Isotopic Steroid** | **Empirical Expected**  % CV  % Recovery | **Blood (3 µL)**  % CV  % Recovery | **Brain (1.47-2.21 mg)**  % CV  % Recovery |
| Cortisol-d4 | NA  100 | 18.2  11.0 ± 0.84 | 10.9  7.71 ± 0.86 |
| Oestradiol-d4 | NA  100 | NA  2.28 ± 0.68 | NA  9.13 ± 0.87 |
| Testosterone-c3 | NA  100 | 10.9  27.5 ± 1.16 | 9.70  23.6 ± 0.84 |
| Dehydroepiandrosterone-d6 | NA  100 | 4.65  21.3 ± 1.20 | 16.7  16.1 ± 0.57 |
| Progesterone-d9 | NA  100 | 25.8  35.1 ± 1.45 | 28.5  19.2 ± 1.19 |

Coefficients of variability were determined using pooled blood and brain tissue samples from male Siberian hamsters, and all samples were analyzed in the same batch. Percent recoveries are presented as mean ± SEM (blood: *n* = 6, brain: *n* = 8) and were calculated relative to the empirical expected sample, which contained 100 pg cortisol-d4, 200 pg oestradiol-d4, 100 pg testosterone-c3, 100 pg dehydroepiandrosterone-d6, and 100 pg progesterone-d9 in 20% acetonitrile with 0.1% formic acid. The empirical expected sample was run alongside blood and brain tissue samples during LC-MS/MS analysis. *Note: coefficients of variability were not available for oestradiol-d4, since levels of this steroid were frequently below the limit of detection for the assay.*

**Table S4.** Summary of multivariate statistical analyses performed to examine the effects of photoperiodic treatment and melatonin administration on body mass, reproductive physiology, and social behaviour in male hamsters.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Statistical Test** | **Dependent Variables** | ***F*** | **df** | **η2 or R2** | ***P*** | ***\**** | **Figure or Table** |
| **Body Mass** | One-Way MANOVA |  | 5.447 | 2,35 | 0.237 | **<0.001** | \*\*\* |  |
|  |  | Body Mass | 5.901 | 2,35 |  | **0.006** | \*\* | Table 1 |
|  |  | Percent Change in Body Mass | 15.60 | 2,35 |  | **<0.001** | \*\*\* | Table 1 |
| **Reproductive Physiology** | PERMANOVA |  | 163.0 | 1,36 | 0.819 | **<0.001** | \*\*\* |  |
|  | Paired Testes Mass |  |  |  |  |  | Table 1 |
|  |  | EWAT Mass |  |  |  |  |  | Table 1 |
| **Aggression** | PERMANOVA |  | 3.260 | 1,36 | 0.083 | ***0.075*** | # |  |
|  |  | Number of Attacks |  |  |  |  |  |  |
|  |  | Attack Duration |  |  |  |  |  | Fig. 1(a) |
|  |  | Latency to First Attack |  |  |  |  |  | Fig. 1(b) |
|  |  | Number of Chases |  |  |  |  |  | Fig. 1(c) |
|  |  | Chase Duration |  |  |  |  |  | Fig. 1(d) |
| **Investigation** | One-Way MANOVA |  | 0.860 | 2,32 | 0.103 | 0.555 | NS |  |
|  |  | NTN Frequency | 0.614 | 2,32 |  | 0.548 | NS |  |
|  |  | NTN Duration | 1.005 | 2,32 |  | 0.377 | NS |  |
|  |  | AGI Frequency | 1.875 | 2,32 |  | 0.170 | NS |  |
|  |  | AGI Duration | 2.449 | 2,32 |  | 0.102 | NS |  |
| **Scent Marking** | PERMANOVA |  | 16.05 | 1,36 | 0.308 | **<0.001** | \*\*\* |  |
|  |  | Scent Marking Frequency |  |  |  |  |  | Fig. 1(e) |
|  |  | Scent Marking Duration |  |  |  |  |  | Fig. 1(f) |
| **Grooming** | One-Way MANOVA |  | 0.499 | 2,35 | 0.028 | 0.736 | NS |  |
|  |  | Grooming Frequency | 0.134 | 2,35 |  | 0.875 | NS | Fig. 1(g) |
|  |  | Grooming Duration | 0.371 | 2,35 |  | 0.693 | NS | Fig. 1(h) |

One-way multivariate analyses of variance (MANOVAs) and non-parametric permutational multivariate analyses of variance (PERMANOVAs) were used to assess the effects of treatment with long-day (LD) photoperiods, LD photoperiods and timed melatonin (M) injections, and short-day (SD) photoperiods on body mass, reproductive physiology, and aggressive, investigative, scent marking, and self-grooming behaviours in male hamsters (LD: *n* =8-9, LD-M: *n* = 12-13, SD: *n* =15-16). *F* statistics (*F*), degrees of freedom (df), estimations of effect size (partial η2 for MANOVAs, R2 for PERMANOVAs), *P*-values (*P*), statistical significance (\*), and the location of figures or tables associated with each analysis are shown. For tests that either showed a significant effect of treatment (*P* < 0.05, in **bold**) or trended towards a significant effect of treatment (*P* < 0.10, in **bold** and *italics*), univariate analyses of variance [one-way analyses of variance (ANOVAs) or non-parametric Kruskal-Wallis one-way ANOVAs on ranks] and post-hoc testing (Tukey’s HSD tests for one-way ANOVAs or Dunn’s tests for multiple comparisons for Kruskal-Wallis one-way ANOVAs on ranks) were conducted to examine pairwise comparisons. *Abbreviations: anogenital investigation, AGI; epididymal white adipose tissue, EWAT; nose-to-nose investigation, NTN.* Symbols: NS (not significant, *P* > 0.10), #*P* < 0.10, \*\**P* < 0.01, \*\*\**P* < 0.001. *Outliers excluded from statistical analysis: one LD-M animal and one SD animal for attack duration, one LD-M animal and one SD animal for anogenital investigation frequency, and one LD animal and one LD-M animal for anogenital investigation duration.*

**Table S5.** Summary of multivariate statistical analyses performed to examine the effects of photoperiodic treatment and melatonin administration on circulating and neural steroid levels in male hamsters.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Statistical Test** | **Dependent Variables** | ***F*** | **df** | **η2 or R2** | ***P*** | ***\**** | **Figure** |
| **Circulating Steroids** | PERMANOVA |  | 2.872 | 1,19 | 0.131 | ***0.091*** | # |  |
|  |  | PROG |  |  |  |  |  | Fig. 3(a) |
|  |  | DHEA |  |  |  |  |  | Fig. 3(b) |
|  |  | T |  |  |  |  |  | Fig. 3(c) |
|  |  | E2 |  |  |  |  |  | Fig. 3(d) |
|  |  | CORT |  |  |  |  |  | Fig. 3(e) |
| **Neural PROG** | PERMANOVA |  | 1.472 | 1,20 | 0.069 | 0.226 | NS |  |
|  |  | LS |  |  |  |  |  | Fig. 4(b) |
|  |  | AH |  |  |  |  |  | Fig. 4(b) |
|  |  | MeA |  |  |  |  |  | Fig. 4(b) |
|  |  | PAG |  |  |  |  |  | Fig. 4(b) |
| **Neural DHEA** | One-Way MANOVA |  | 1.067 | 2,16 | 0.234 | 0.413 | NS |  |
|  |  | LS | 1.365 | 2,16 |  | 0.287 | NS | Fig. 4(c) |
|  |  | AH | 0.369 | 2,16 |  | 0.698 | NS | Fig. 4(c) |
|  |  | MeA | 1.212 | 2,16 |  | 0.327 | NS | Fig. 4(c) |
|  |  | PAG | 2.848 | 2,16 |  | ***0.094*** | # | Fig. 4(c) |
| **Neural T** | PERMANOVA |  | 17.47 | 1,20 | 0.466 | **0.002** | \*\* |  |
|  |  | LS |  |  |  |  |  | Fig. 4(d) |
|  |  | AH |  |  |  |  |  | Fig. 4(d) |
|  |  | MeA |  |  |  |  |  | Fig. 4(d) |
|  |  | PAG |  |  |  |  |  | Fig. 4(d) |
| **Neural E2** | PERMANOVA |  | 4.445 | 1,20 | 0.182 | **0.036** | \* |  |
|  |  | LS |  |  |  |  |  | Fig. 4(e) |
|  |  | AH |  |  |  |  |  | Fig. 4(e) |
|  |  | MeA |  |  |  |  |  | Fig. 4(e) |
|  |  | PAG |  |  |  |  |  | Fig. 4(e) |
| **Neural CORT** | One-Way MANOVA |  | 0.709 | 2,12 | 0.221 | 0.681 | NS |  |
|  |  | LS | 0.906 | 2,12 |  | 0.430 | NS | Fig. 4(f) |
|  |  | AH | 0.742 | 2,12 |  | 0.497 | NS | Fig. 4(f) |
|  |  | MeA | 0.212 | 2,12 |  | 0.812 | NS | Fig. 4(f) |
|  |  | PAG | 1.271 | 2,12 |  | 0.316 | NS | Fig. 4(f) |

One-way multivariate analyses of variance (MANOVAs) and non-parametric permutational multivariate analyses of variance (PERMANOVAs) were used to assess the effects of treatment with long-day (LD) photoperiods, LD photoperiods and timed melatonin (M) injections, and short-day (SD) photoperiods on circulating and neural steroid hormone levels in male hamsters (LD: *n* = 4-6, LD-M: *n* = 6-8, SD: *n* = 6-8). *F* statistics (*F*), degrees of freedom (df), estimations of effect size (partial η2 for MANOVAs, R2 for PERMANOVAs), *P*-values (*P*), statistical significance (\*), and the location of figures associated with each analysis are shown. For tests that either showed a significant effect of treatment (*P* < 0.05, in **bold**) or trended towards a significant effect of treatment (*P* < 0.10, in **bold** and *italics*), univariate analyses of variance [one-way analyses of variance (ANOVAs) or non-parametric Kruskal-Wallis one-way ANOVAs on ranks] and post-hoc testing (Tukey’s HSD tests for one-way ANOVAs or Dunn’s tests for multiple comparisons for Kruskal-Wallis one-way ANOVAs on ranks) were conducted to examine pairwise comparisons. *Abbreviations: anterior hypothalamus, AH; cortisol, CORT; dehydroepiandrosterone, DHEA; oestradiol, E2; lateral septum, LS; medial amygdala, MeA; periaqueductal gray, PAG; progesterone, PROG; testosterone, T.* Symbols: NS (not significant, *P* > 0.10), #*P* < 0.10, \**P* < 0.05, \*\**P* < 0.01. *Outliers excluded from statistical analysis: one LD-M animal for circulating T concentration, one LD-M animal for PROG concentration in the LS, one SD animal for DHEA concentration in the PAG, and one LD-M animal for T concentration in the AH.*

**Table S6.** Correlations between aggressive behaviour, circulating steroid levels, and neurosteroid levels in male hamsters.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Steroid** | **LD** | | **LD-M** | | **SD** | |
| ρ | *P* | ρ | *P* | ρ | *P* |
| **Number of Attacks** | AH E2 | -0.06 | 0.91 | 0.18 | 0.68 | -0.64 | ***0.09*** |
| PAG DHEA | 0.87 | ***0.05*** | 0.08 | 0.84 | -0.21 | 0.64 |
| PAG E2 | -0.41 | 0.49 | 0.34 | 0.41 | -0.64 | ***0.09*** |
| **Attack Duration** | Circulating PROG | 0.77 | ***0.07*** | -0.23 | 0.61 | -0.14 | 0.76 |
| LS PROG | 0.77 | ***0.07*** | 0.00 | 1.00 | -0.39 | 0.38 |
| AH PROG | 0.75 | ***0.08*** | 0.07 | 0.87 | 0.43 | 0.34 |
| AH DHEA | 0.81 | ***0.05*** | -0.20 | 0.63 | -0.36 | 0.43 |
| MeA PROG | 0.60 | 0.28 | -0.43 | 0.29 | 0.75 | ***0.08*** |
| MeA CORT | 0.60 | 0.40 | -0.68 | ***0.09*** | 0.20 | 0.70 |
| PAG CORT | -0.20 | 0.80 | -0.86 | **0.01** | 0.30 | 0.62 |
| **Latency to First Attack** | Circulating PROG | -0.77 | ***0.07*** | -0.20 | 0.70 | -0.41 | 0.32 |
| Circulating DHEA | -0.49 | 0.33 | 0.60 | 0.21 | -0.63 | ***0.09*** |
| Circulating E2 | 0.03 | 0.95 | 0.21 | 0.69 | 0.64 | ***0.09*** |
| LS PROG | -0.77 | ***0.07*** | -0.20 | 0.70 | -0.02 | 0.96 |
| LS DHEA | -0.31 | 0.54 | 0.31 | 0.54 | -0.69 | ***0.06*** |
| AH PROG | -0.93 | **0.01** | -0.07 | 0.88 | -0.26 | 0.53 |
| AH T | -0.26 | 0.62 | 0.77 | ***0.07*** | -0.11 | 0.80 |
| MeA PROG | -0.90 | **0.04** | 0.54 | 0.22 | -0.37 | 0.41 |

Spearman’s rank correlations between aggressive behaviour, circulating steroid levels, and neurosteroid levels in long day animals (LD), LD animals administered timed melatonin injections (LD-M), and animals that were exposed to short-day photoperiods (SD). Correlation coefficients (ρ) and *P*-values (*P*) are shown for each analysis, which was performed within each treatment group (LD: *n* = 4-6, LD-M: *n* = 6-8, SD: *n* = 6-8). Only correlations that were significant (*P* < 0.05, in **bold**) or trended towards significance (*P* < 0.10, in **bold** and *italics*) in at least one treatment group are shown. *Abbreviations: anterior hypothalamus, AH; cortisol, CORT; dehydroepiandrosterone, DHEA; oestradiol, E2; lateral septum, LS; medial amygdala, MeA; periaqueductal gray, PAG; progesterone, PROG; testosterone, T.*

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