**ELECTRONIC SUPPLEMENTARY MATERIAL**

**Sex-specific endocrine regulation of seasonal aggression in Siberian hamsters**

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**METHODS**

***Seasonal phenotypes***

Body mass was measured weekly throughout the study, and reproductive tissues (testes for males, ovaries for females) were collected and weighed after 10 weeks of photoperiodic treatment and injections. These two measures were the primary criteria used to classify hamsters as responsive or non-responsive to photoperiodic treatment. For all hamsters in the LD and SD groups, each of the two variables used for classification agreed. LD hamsters had functional reproductive tissues (i.e., males had a paired testes mass between 0.690-0.890 g, females had a paired ovarian mass between 9-24 mg) and displayed no significant change in body mass. By contrast, LD-M and SD hamsters that were responsive to photoperiodic treatment had regressed reproductive tissues [i.e., had a paired testes mass (for males) or a paired ovarian mass (for females) that was > 2 standard deviations below the average mass of LD hamsters] and showed a significant change in body mass [> 4%; percent change in body mass (means ± SEM) – LD-M males: -14.1 ± 1.43%, SD males: -7.43 ± 1.28%, LD-M females: -12.9 ± 2.24%, SD females: -8.09 ± 1.00%].

***Behavioural testing***

Behavioural trials for the resident-intruder paradigm were performed within the first 3 h of the dark phase. Staged dyads were formed, which were composed of an experimental (i.e., resident) animal 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 placed 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 territory (1, 2), for a period of 5 min. 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 13-15 trials). If an intruder was used twice during a single testing period (*n* = 5 occurrences), the behaviour trials for which this hamster was used were separated by ≥ 1.5 h. Hamsters used as intruders (males: *n* = 14, females: *n* = 14) were housed in pairs with a same-sex sibling and maintained in LDs prior to behavioural testing and throughout the study.

Behavioural interactions were video recorded and scored for aggressive and non-aggressive (i.e., investigation, self-grooming) behaviours by a single observer (K.M.M.) who was blind to the experimental conditions. Measures of aggression, investigation, and grooming were defined according to prior studies on same-sex social behaviour in male and female Siberian hamsters (3, 4). Eight hamsters (males – LD: *n* = 2, LD-M: *n* = 2, SD: *n* = 3; females – LD: *n* = 1) did not display attacking behaviour during the testing period and were assigned an attack latency of 300 s for the purpose of statistical analysis. For the principal component analyses (PCAs) used to generate a composite ‘aggression score’ (PCAgg) and a composite ‘investigation score’ (PCInv), the set of variables included in each PCA was evaluated for suitability of factor analysis with the Kaiser-Meyer-Olkin measure of sampling adequacy and Bartlett's test of sphericity (5) using the *KMO* and *cortest.bartlett* functions of the *psych* package in R version 4.1.2 (6, 7). The data that comprised each PCA had a Kaiser-Meyer-Olkin measure of sampling adequacy > 0.500 and a significant *p*-value (*p* < 0.05) for Bartlett’s test of sphericity and, thus, were considered appropriate for factor analysis (8, 9) (PCAgg – Kaiser-Meyer-Olkin measure of sampling adequacy = 0.650, Bartlett’s test of sphericity: χ2 = 180.613, d.f. = 10, *p* < 0.001; PCInv – Kaiser-Meyer-Olkin measure of sampling adequacy = 0.540, Bartlett’s test of sphericity: χ2 = 120.840, d.f. = 6, *p* < 0.001). PCAs were conducted using the *prcomp* function of the *stats* package (6), and data were standardized using Z-scores to account for differences in scaling between variables (10).

***Tissue collection and processing***

Following behavioural testing, hamsters were euthanized with a lethal intraperitoneal injection (0.3 mL) of ketamine (150 mg/kg) and xylazine (30 mg/kg) cocktail in 0.9% saline. Adrenal glands were rapidly extracted (≤ 5 min following euthanasia), placed in polypropylene microtubes (Sarstedt Inc., Nümbrecht, Germany) containing 5 zirconium ceramic beads (1.4 mm diameter; Fisher Scientific, Waltham, WA, USA), flash frozen on dry ice, and stored at -80°C. Whole brains were rapidly extracted (≤ 5 min following euthanasia), flash frozen on crushed dry ice, and stored at -80˚C until sectioning. Following sectioning and microdissection, brain punches were placed in polypropylene microtubes containing 5 zirconium ceramic beads and stored at -80°C. In addition, reproductive tissues (testes for males, ovaries for females) were removed, weighed, and used to classify seasonal phenotypes (see “***Seasonal Phenotypes***” section above and in the Methods section of the main text).

***In vitro measurement of 3β-HSD activity***

*Tissue homogenate preparation and incubation assay*

Prior to the incubation assay, adrenal and brain tissues were homogenized in 200 µL cold sucrose phosphate buffer (100 mM sodium phosphate buffer, 1.5 mM EDTA, and 20% glycerol at pH 7.4) containing four freshly added protease inhibitors [phenylmethylsulfonyl fluoride (1 mM), peptastin A (5 µg/mL), antipain (5 µg/mL), and leupeptin (5 µg/mL)]. Following homogenization, samples were aliquoted into three tubes: 1) a protein sample (10 µL), which was used to measure total protein content (see “***Protein Content”*** section below), 2) a pre-incubation assay sample (adrenals: 7.5 µL, brain: 95 µL), which was used to measure baseline concentrations of progesterone (PROG)-c2d2 and androstenedione (AE)-d2, and 3) a post-incubation assay sample (adrenals: 7.5 µL, brain: 95 µL), which was used to measure post-incubation concentrations of PROG-c2d2 and AE-d2. Protein and pre-incubation assay samples were flash frozen on dry ice and stored at -80°C, and all steps prior to freezing (for protein and pre-incubation assay samples) or incubation (for post-incubation assay samples) were carried out on wet ice. Control samples, which contained all components except for supernatant, were incubated alongside experimental samples and were used to confirm a lack of 3β-HSD enzymatic activity in the absence of adrenal and brain tissue.

*Protein content*

Total protein content was determined using a commercially available Bradford assay kit (Pierce™ Coomassie Plus Bradford Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA; micro microplate protocol, working range: 1-25 µg/mL). The validity of the assay was determined by comparing Siberian hamster adrenal and brain samples of varying dilutions with a standard curve generated using bovine serum albumin reference standards provided by the kit. Brain samples were diluted 1:40 and adrenal samples were diluted 1:200 or 1:500 in incubation assay buffer (100 mM sodium phosphate buffer, 1.5 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/mL pepstatin A, antipain, and leupeptin at pH 7.4) that was diluted 1:2 with ultrapure water. Samples were run in duplicate per the manufacturer’s instructions using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), and all samples contained protein concentrations that were within the working range of the assay. Tissue samples from different treatment groups were counterbalanced across six 96-well plates, and adrenal and brain samples from the same individual were run on the same plate. Samples with a coefficient of variability (CV) greater than 20% were re-analysed. Inter-assay and intra-assay CVs were determined using pooled adrenal gland and brain samples from LD hamsters. The inter-assay variability was ≤ 11.23% (adrenals: 11.23%, brain: 9.21%), and the intra-assay variability was ≤ 3.59% (adrenals: 1.52 ± 0.12%, brain: 3.59 ± 0.67%).

*Validations of 3β-HSD activity assay*

A set of validation studies was performed to optimize the reaction conditions and confirm the efficacy of the 3β-HSD incubation assay. A time course study was conducted to determine the appropriate duration for incubations, in which pooled adrenal and brain supernatants were incubated for 15 min, 30 min, 60 min (1 h), 180 min (3 h), or 360 min (6 h). We determined that PROG-c2d2 and AE-d2 production via 3β-HSD peaked at 3 h for adrenal samples, whereas PROG and AE synthesis peaked at 6 h for brain samples (electronic supplementary material, figure S1); thus, we selected an incubation period of 3 h for adrenal samples and an incubation period of 6 h for brain samples. Furthermore, we determined whether adding abiraterone acetate and fadrozole hydrochloride (pharmacological inhibitors of cytochrome P450c17 and aromatase, respectively) decreased the metabolism of formed PROG-c2d2 and AE-d2. Because PROG and AE synthesis was significantly lower in adrenal and brain samples that did not contain these inhibitors relative to control samples (Welch’s t-tests, *p* ≤ 0.025 for all comparisons; electronic supplementary material, table S2), abiraterone acetate and fadrozole hydrochloride were included in the incubation assay. Finally, we confirmed that trilostane, a pharmacological inhibitor of 3β-HSD, significantly reduced PROG-c2d2 and AE-d2 production in adrenal and brain samples compared to control samples (Welch’s t-tests, *p* ≤ 0.031 for all comparisons; electronic supplementary material, figure S2).

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

In addition to isotopically labelled analogues of PROG and AE (PROG-d9 and AE-d7), 5 ng of pregnanediol in 20% acetonitrile with 0.1% formic acid was spiked into each sample to mitigate potential stickiness of isotopically labelled and endogenous steroids for plastics and glass used in the steroid extraction procedure. Samples were centrifuged at 16,100 x *g* for 10 min at 4°C, and supernatant (adrenals: 20 µL, brain: 100 µL) was collected into 16 x 100 mm glass test tubes stored on wet ice. The centrifugation process was repeated after adding 100 µL 100% acetonitrile with 0.1% formic acid to each sample, and 100 µL of the supernatants was transferred to their respective glass test tubes. Samples were then placed in a water bath, dried at 35°C for 60 min using an Evap-O-Rac system (Cole-Parmer, Vernon Hills, IL, USA), and reconstituted in 100 µL 20% methanol with 0.1% formic acid. 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. 100 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 100 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). In addition, 10 µL of isotopically labelled steroids, which contained 5 µL of 1 µM PROG-d9 and 5 µL of 1 µM AE-d7 in 20% acetonitrile with 0.1% formic acid, was added directly into an amber vial fitted with a glass vial insert. This sample, which represented the empirical expected concentrations of isotopically labelled analogues of PROG and AE, was run alongside tissue samples and was used to track percent recovery for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) protocol. All samples were dried in a CentriVap vacuum concentrator (Labconco, Kansas City, MO, USA), reconstituted in 20 µL 20% acetonitrile with 0.1% formic acid, sealed with crimp top caps (11 mm diameter; Agilent Technologies, Santa Clara, CA, USA), and stored at -20˚C.

***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; Thermo Fisher 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 min gradient was performed as follows: 0 to 0.5 min, 15% to 50% solvent B; 0.5 to 16 min, 50% to 100% solvent B; 16 to 22 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 the Orbitrap Fusion Lumos mass spectrometer 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. PROG-c2d2 and AE-d2 concentrations were calculated using isotopic internal standard quantification, in which the amount of the isotopically labelled analogue for each steroid (PROG-d9 and AE-d7, respectively) was multiplied by the ratio of the fragment ion signal for the steroid of interest (i.e., PROG-c2d2 or AE-d2) to the fragment ion signal for their corresponding isotopically labelled analogue (i.e., PROG-d9 or AE-d7) (11, 12).

***Statistical analyses***

Statistical outliers were examined with Grubbs’ Tests using the *grubbs.test* function of the *outliers* package (13), and data points that affected the conceptual conclusions of the study were excluded from statistical analysis (stated in the footnotes of the statistical analysis summary tables in the electronic supplementary material). 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* package (14). For univariate analyses, normality of linear model residuals was assessed with Shapiro-Wilk tests using the *shapiro.test* function of the *stats* package (6), and homogeneity of variances was assessed with Levene’s tests using the *leveneTest* function of the *car* package (15). Data that exhibited a non-normal distribution were visualized with Cullen and Frey plots using the *descdist* function of the *fitdistrplus* package, and the best-fit model was selected on the basis of Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values generated using the *gofstat* function of the *fitdistrplus* package (16).

For each model, *R*2 values (for two-way MANOVAs and ANOVAs) and Wald χ2 values (for GLMs) were calculated to assess goodness of fit, and *R*2 (for PERMANOVAs), partial η2 (for two-way MANOVAs and ANOVAs), and Nagelkerke's pseudo-*R*2 values (for GLMs) were determined to estimate effect size (presented in the main text and electronic supplementary material) (17, 18). For estimations of effect size, 0.04-0.25 indicates a small effect, 0.25-0.64 indicates a moderate effect, and > 0.64 indicates a strong effect (19). Data were analysed using the *adonis* function of the *vegan* package (PERMANOVAs) (20), the *lm and manova* functions of the *stats* package (ANOVAs and MANOVAs, respectively) (6), the *glht* function of the *multcomp* package (Tukey’s HSD post-hoc tests) (21), the *dunn.test* function of the *dunn.test* package (Dunn’s post-hoc tests) (22), the *summ* function of the *jtools* package (Wald χ2 and Nagelkerke's pseudo-*R*2 values) (23), and the *etasq* function of the *heplots* package (partial η2 values) (24).

**TABLES**

**Table S1.** Principal component loading values and eigenvalues for variables that were used to generate composite aggression and investigation scores via principal component analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| **Variables** | **PCAgg** | **Variables** | **PCInv** |
| Number of Attacks | **0.506** | NTN Frequency | **0.532** |
| Attack Duration | **0.426** | NTN Duration | **0.492** |
| Number of Chases | **0.468** | AGI Frequency | **0.539** |
| Chase Duration | **0.460** | AGI Duration | **0.429** |
| Latency to First Attack | **-0.363** |  |  |
| **Eigenvalues** | 3.160 |  | 2.543 |

Composite aggression and investigation scores (PCAgg and PCInv, respectively) were used to determine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on aggressive and investigative social behaviours. PCAgg accounted for 63.2% of the total variance, and PCInv accounted for 63.6% of the total variance. **Bold** values indicate variables that loaded strongly (< -0.3 or > 0.3) onto a given principal component. *Abbreviations: AGI, anogenital investigation; NTN, nose-to-nose investigation.*

**Table S2**. MS/MS transitions for internal standards and endogenous and isotopically labelled steroid hormones in Siberian hamster adrenal and brain tissue.

|  |  |  |  |
| --- | --- | --- | --- |
| **Steroid** | **Retention Time (min)** | **Mass-to-Charge Ratio (m/z)** | |
| **Precursor** | **Fragment** |
| Dehydroepiandrosterone-d2 | 16.0 | 291.2 | 255.208 |
| Androstenedione | 16.1 | 287.2 | 97.065, 109.065 |
| Androstenedione-d2 | 16.1 | 289.2 | 97.065, 109.065 |
| Androstenedione-d7 | 16.1 | 294.2 | 100.084, 113.090 |
| Pregnenolone-c2d2 | 20.9 | 321.3 | 285.246, 303.257 |
| Progesterone | 21.2 | 315.2 | 97.065, 109.065 |
| Progesterone-c2d2 | 21.2 | 319.2 | 97.065, 109.065 |
| Progesterone-d9 | 21.2 | 324.2 | 100.084, 113.090 |

Retention times and mass-to-charge ratios of precursor and fragment ions are shown for each internal standard and endogenous and isotopically labelled steroid hormone. 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 H*** of Methods).

**Table S3.** Accuracy and precision measurements of internal standards in Siberian hamster tissue for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| **Isotopic Steroid** | **Empirical Expected**  Inter-assay %CV  Intra-assay %CV  % Recovery | **Adrenal Glands**  Inter-assay %CV  Intra-assay %CV  % Recovery | **Brain**  Inter-assay %CV  Intra-assay %CV  % Recovery |
| Progesterone-d9 | NA  NA  100 | 10.48  6.213 ± 2.030  23.16 ± 5.552 | 12.72  8.073 ± 0.798  4.791 ± 0.573 |
| Androstenedione-d7 | NA  NA  100 | 11.68  6.119 ± 1.866  38.84 ± 5.199 | 12.42  5.215 ± 2.444  21.73 ± 2.751 |

Coefficients of variability (CV) were determined using pooled adrenal and hypothalamic (brain) tissue samples, and samples were analysed either across batches (for inter-assay %CV calculations; adrenals: *n* = 3, brain: *n* = 3) or within the same batch (for intra-assay %CV calculations, presented as means ± SEM; adrenals: *n* = 7-8, brain: *n* = 6). Percent recoveries are presented as means ± SEM (adrenals: *n* = 16, brain: *n* = 14) and were calculated relative to the empirical expected sample, which contained 50 nM androstenedione-d7 and 50 nM progesterone-d9 in 20% acetonitrile with 0.1% formic acid. The empirical expected sample was run alongside tissue samples during LC-MS/MS analysis.

**Table S4.** Precision measurements for 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase (3β-HSD) enzymatic activity assay in Siberian hamster adrenal and brain tissue.

|  |  |  |
| --- | --- | --- |
| **Measurement** | **Adrenal Glands**  Inter-assay %CV  Intra-assay %CV | **Brain**  Inter-assay %CV  Intra-assay %CV |
| 3β-HSD Activity, PROG Synthesis  (nmol PROG-c2d2/mg protein) | 12.70  3.531 ± 1.053 | 11.03  7.902 ± 0.696 |
| 3β-HSD Activity, AE Synthesis  (nmol AE-d2/mg protein) | 15.62  4.821 ± 1.102 | 15.97  5.694 ± 0.583 |

Coefficients of variability (CV) were determined using pooled adrenal and hypothalamic (brain) tissue samples, and samples were analysed either across batches (for inter-assay %CV calculations; adrenals: *n* = 3, brain: *n* = 3) or within the same batch (for intra-assay %CV calculations, presented as means ± SEM; adrenals: *n* = 4, brain: *n* = 4-6). *Abbreviations: AE, androstenedione; PROG, progesterone.*

**Table S5**. Timed melatonin injections and short-day photoperiods reduced body and reproductive tissue mass in male and female hamsters.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Males** | | | **Females** | | | ***F-* or *H-Statistic*** | **df** | ***p*** |
|  | **LD** | **LD-M** | **SD** | **LD** | **LD-M** | **SD** |
| Percent Change in Body Mass | -4.03 ± 0.99a | -14.1 ± 1.43**b** | -7.43 ± 1.28**b** | -2.14 ± 5.29a | -12.9 ± 2.24**b** | -8.09 ± 1.00a,b | 4.180 | 5,38 | **0.004** |
| Paired Testes Mass (g) | 0.77 ± 0.03a | 0.26 ± 0.04**b** | 0.39 ± 0.09**b** | --- | --- | --- | 21.41 | 2,18 | **<0.001** |
| Paired Ovarian Mass (mg) | --- | --- | --- | 15.3 ± 2.06a | 10.5 ± 1.73**b** | 6.20 ± 1.88**b** | 4.288 | 2,16 | **0.032** |

Means ± SEM (males – LD: *n* = 6, LD-M: *n* = 8, SD: *n* = 7-8; females – LD: *n* = 6, LD-M: *n* = 8, SD: *n* = 5-8) of percent change in body mass, paired testes mass, and paired ovarian mass in long-day hamsters (LD), LD hamsters administered timed melatonin injections (LD-M), and short-day hamsters (SD) following 10 weeks of treatment. Significant *p*-values are shown in **bold**, and different letters indicate a significant difference between treatment groups within each sex (*p* < 0.05; percent change in body mass: two-way ANOVA with Tukey’s HSD post-hoc tests; paired testes mass and paired ovarian mass: one-way ANOVAs with Tukey’s HSD post-hoc tests). *Outliers excluded from statistical analysis: one SD male for paired testes mass and two SD females for paired ovarian mass.*

**Table S6.** Summary of multivariate statistical analyses performed to examine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on social behaviour in male and female hamsters.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Statistical Test** | **Dependent Variables** | **Factors** | ***F*** | **df** | ***R*2** | ***p*** | ***\**** |
| **Aggression** | PERMANOVA | Number of Attacks | Treatment | 4.928 | 1,42 | 0.105 | **0.026** | \* |
|  |  | Attack Duration | Sex | 1.044 | 1,42 | 0.024 | 0.306 | NS |
|  |  | Latency to First Attack | Treatment\*Sex | 3.044 | 2,41 | 0.129 | ***0.056*** | # |
|  |  | Number of Chases |  |  |  |  |  |  |
|  |  | Chase Duration |  |  |  |  |  |  |
|  |  | AGG Frequency |  |  |  |  |  |  |
|  |  | AGG Duration |  |  |  |  |  |  |
|  |  | AGG Score |  |  |  |  |  |  |
|  |  | PCAgg |  |  |  |  |  |  |
| **Investigation** | PERMANOVA | NTN Frequency | Treatment | 0.294 | 1,42 | 0.007 | 0.720 | NS |
|  |  | NTN Duration | Sex | 0.893 | 1,42 | 0.021 | 0.393 | NS |
|  |  | AGI Frequency | Treatment\*Sex | 0.585 | 2,41 | 0.028 | 0.631 | NS |
|  |  | AGI Duration |  |  |  |  |  |  |
|  |  | INV Frequency |  |  |  |  |  |  |
|  |  | INV Duration |  |  |  |  |  |  |
|  |  | PCInv |  |  |  |  |  |  |
| **Grooming** | PERMANOVA | GR Frequency | Treatment | 0.379 | 1,42 | 0.009 | 0.546 | NS |
|  |  | GR Duration | Sex | 1.277 | 1,42 | 0.030 | 0.271 | NS |
|  |  |  | Treatment\*Sex | 0.820 | 2,41 | 0.038 | 0.478 | NS |

Permutational multivariate analyses of variance (PERMANOVAs) were used to assess the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on aggressive, investigative, and self-grooming behaviours in male and female hamsters (males – LD: *n* = 5-6, LD-M: *n* = 8, SD: *n* = 8; females – LD: *n* = 5-6, LD-M: *n* = 7-8, SD: *n* = 8). *F*-statistics (*F*), degrees of freedom (df), estimations of effect size (*R*2), *p*-values (*p*), and statistical significance (\*) for each analysis are shown. For tests that either showed a significant effect (*p* < 0.05, in **bold**) or trended towards a significant effect (*p* < 0.10, in **bold** and *italics*) of treatment, sex, and/or the interaction between treatment and sex, univariate analyses of variance [two-way analyses of variance (ANOVAs) or generalized linear models (GLMs)] and post-hoc testing (Tukey’s HSD tests for two-way ANOVAs or Dunn’s tests for GLMs) were conducted to examine pairwise comparisons. *Abbreviations: AGG, aggression; AGI, anogenital investigation; GR, grooming; INV, investigation; NTN, nose-to-nose investigation; PCAgg, composite aggression score; PCInv, composite investigation score.* Symbols: NS (not significant, *p* > 0.10), #*p* < 0.10, \**p* < 0.05. *Outliers excluded from statistical analysis: one LD male and one LD female for number of attacks, one LD male and one LD-M female for attack duration, one LD male and one LD female for AGG frequency, one LD male and one LD-M female for AGG duration, one LD female for AGG score, and one LD male for PCAgg.*

**Table S7.** Summary of univariate statistical analyses performed to examine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on aggressive behaviour in male and female hamsters.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Statistical Test and Family** | **Coefficients** | **Estimate ± SE** | ***t or z*** | ***p*** | ***\**** |
| **Number of Attacks** | GLM, Poisson | Treatment | 0.504 ± 0.109 | 4.623 | **<0.001** | \*\*\* |
|  | (Log link function) | Sex | 0.181 ± 0.501 | 0.362 | 0.718 | NS |
|  |  | Treatment\*Sex | 0.047 ± 0.143 | 0.329 | 0.742 | NS |
| **Attack Duration** | Two-Way ANOVA | Treatment | 10.21 ± 5.418 | 1.885 | ***0.067*** | # |
|  |  | Sex | 14.57 ± 21.11 | 0.604 | 0.549 | NS |
|  |  | Treatment\*Sex | -6.100 ± 7.489 | -0.815 | 0.420 | NS |
| **Latency to First Attack** | Two-Way ANOVA | Treatment | -21.83 ± 36.86 | -0.592 | 0.259 | NS |
|  |  | Sex | -0.005 ± 117.6 | 0.000 | 0.618 | NS |
|  |  | Treatment\*Sex | 174.8 ± 83.16 | 2.101 | **0.026** | \* |
| **Number of Chases** | GLM, Poisson | Treatment | -1.616 ± 1.193 | -1.354 | 0.176 | NS |
|  | (Log link function) | Sex | 0.073 ± 0.271 | 0.269 | 0.788 | NS |
|  |  | Treatment\*Sex | 0.686 ± 0.353 | 1.941 | ***0.052*** | # |
| **Chase Duration** | GLM, Binomial | Treatment | 1.568 ± 2.993 | 0.524 | 0.600 | NS |
|  | (Logit link function) | Sex | -0.086 ± 0.901 | -0.096 | 0.924 | NS |
|  |  | Treatment\*Sex | 0.682 ± 0.704 | 0.969 | 0.333 | NS |
| **AGG Frequency** | GLM, Poisson | Treatment | 0.439 ± 0.101 | 4.356 | **<0.001** | \*\*\* |
|  | (Log link function) | Sex | -0.238 ± 0.468 | -0.509 | 0.611 | NS |
|  |  | Treatment\*Sex | 0.178 ± 0.134 | 1.329 | 0.184 | NS |
| **AGG Duration** | Two-Way ANOVA | Treatment | 10.23 ± 5.642 | 1.813 | ***0.078*** | # |
|  |  | Sex | 12.70 ± 25.10 | 0.506 | 0.616 | NS |
|  |  | Treatment\*Sex | -5.370 ± 7.797 | -0.689 | 0.495 | NS |
| **AGG Score** | GLM, Poisson | Treatment | 0.310 ± 0.088 | 3.516 | **<0.001** | \*\*\* |
|  | (Log link function) | Sex | -0.406 ± 0.436 | -0.931 | 0.352 | NS |
|  |  | Treatment\*Sex | 0.146 ± 0.127 | 1.146 | 0.252 | NS |
| **PCAgg** | Two-Way ANOVA | Treatment | -1.431 ± 0.785 | 3.327 | ***0.092*** | # |
|  |  | Sex | -0.149 ± 2.200 | 1.671 | 0.204 | NS |
|  |  | Treatment\*Sex | 0.270 ± 0.684 | 0.155 | 0.696 | NS |

Two-way analyses of variance (ANOVAs) and generalized linear models (GLMs) were used to examine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on aggression in male and female hamsters (males – LD: *n* = 5-6, LD-M: *n* = 8, SD: *n* = 8; females – LD: *n* = 5-6, LD-M: *n* = 7-8, SD: *n* = 8). Coefficient estimates ± standard error (SE), *t*-statistics (*t*, for two-way ANOVAs), *z*-statistics (*z*, for GLMs), *p*-values (*p*), and statistical significance (\*) for each model are shown. **Boldface** indicates *p* < 0.05, whereas **boldface** and *italics* indicate *p* < 0.10. Test statistics (*F*-statistic for two-way ANOVAs and Wald χ2 for GLMs) and estimations of effect size (*R*2 values for two-way ANOVAs and Nagelkerke's pseudo-*R*2 values for GLMs) for each model are provided in the Results section of the main text. *Abbreviations: AGG, aggression; PCAgg, composite aggression score.* Symbols: NS (not significant, *p* > 0.10), #*p* < 0.10, \**p* < 0.05, \*\*\**p* < 0.001. *Outliers excluded from statistical analysis: one LD male and one LD female for number of attacks, one LD male and one LD-M female for attack duration, one LD male and one LD female for AGG frequency, one LD male and one LD-M female for AGG duration, one LD female for AGG score, and one LD male for PCAgg.*

**Table S8.** Treatment with timed melatonin injections and short-day photoperiods increased aggressive behaviour in male and female hamsters.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Males** | | | **Females** | | |
|  | **LD** | **LD-M** | **SD** | **LD** | **LD-M** | **SD** |
| Number of Attacks | 1.800 ± 0.917a | 10.13 ± 3.786**b** | 10.13 ± 4.068**b** | 5.400 ± 2.731a | 10.00 ± 3.262a,b | 16.75 ± 4.624**b** |
| Attack Duration | 3.508 ± 2.190a | 22.94 ± 8.146**b** | 25.47 ± 11.58**b** | 12.10 ± 6.009a | 10.43 ± 3.278a | 19.80 ± 3.492a |
| Latency to First Attack (s) | 169.8 ± 47.51a | 144.5 ± 38.87a | 155.4 ± 44.79a | 146.3 ± 47.39a | 48.88 ± 11.58**b** | 83.13 ± 14.85a,b |
| Number of Chases | 0.000 ± 0.000a | 2.375 ± 1.499**b** | 0.375 ± 0.263a,b | 0.833 ± 0.654a | 1.375 ± 0.653a,b | 3.375 ± 1.647**b** |
| AGG Frequency | 1.800 ± 0.917a | 12.50 ± 5.227**b** | 10.50 ± 4.259**b** | 5.600 ± 2.839a | 11.38 ± 3.798a,b | 20.13 ± 5.696**b** |
| AGG Duration | 3.508 ± 2.190a | 24.26 ± 8.795**b** | 25.72 ± 11.70**b** | 12.32 ± 6.127a | 11.29 ± 3.894a | 21.49 ± 3.658a |
| AGG Score | 5.833 ± 3.591a | 11.25 ± 3.432**b** | 12.13 ± 4.696**b** | 5.600 ± 2.731a | 10.63 ± 3.713a,b | 15.13 ± 3.131**b** |

Means ± SEM (males – LD: *n* = 5-6, LD-M: *n* = 8, SD: *n* = 8; females – LD: *n* = 5-6, LD-M: *n* = 7-8, SD: *n* = 8) of number of attacks, attack duration, latency to first attack, number of chases, aggression (AGG) frequency, AGG duration, and AGG score in long-day hamsters (LD), LD hamsters administered timed melatonin injections (LD-M), and short-day hamsters (SD) following 10 weeks of treatment. Different letters indicate a significant difference between treatment groups within each sex (*p* < 0.05).

**Table S9.** Summary of multivariate statistical analyses performed to examine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase (3β-HSD) activity in the adrenal glands and brain of male and female hamsters.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Statistical Test** | **Dependent Variables** | **Factors** | ***F*** | **df** | **η*2*** | ***p*** | ***\**** |
| **PROG Synthesis** | Two-Way MANOVA | Adrenal Glands | Treatment | 0.159 | 3,30 | 0.032 | 0.923 | NS |
|  |  | AH | Sex | 3.355 | 3,30 | 0.251 | **0.032** | \* |
|  |  | PAG | Treatment\*Sex | 0.573 | 3,30 | 0.054 | 0.637 | NS |
| **AE Synthesis** | Two-Way MANOVA | Adrenal Glands | Treatment | 1.082 | 3,37 | 0.080 | 0.369 | NS |
|  |  | AH | Sex | 7.832 | 3,37 | 0.388 | **<0.001** | \*\*\* |
|  |  | PAG | Treatment\*Sex | 0.080 | 3,37 | 0.006 | 0.971 | NS |
| **AE: PROG Synthesis** | Two-Way MANOVA | Adrenal Glands | Treatment | 1.120 | 3,28 | 0.128 | 0.328 | NS |
|  |  | AH | Sex | 2.945 | 3,28 | 0.183 | ***0.050*** | # |
|  |  | PAG | Treatment\*Sex | 0.798 | 3,28 | 0.079 | 0.506 | NS |

Two-way multivariate analyses of variance (MANOVAs) were used to examine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on adrenal and neural 3β-HSD activity in male and female hamsters (males – LD: *n* = 4-6, LD-M: *n* = 6-8, SD: *n* = 6-8; females – LD: *n* = 4-6, LD-M: *n* = 5-8, SD: *n* = 8). *F*-statistics (*F*), degrees of freedom (df), estimations of effect size (partial η2), *p*-values (*p*), and statistical significance (\*) for each analysis are shown. For tests that either showed a significant effect (*p* < 0.05, in **bold**) or trended towards a significant effect (*p* < 0.10, in **bold** and *italics*) of treatment, sex, and/or the interaction between treatment and sex, two-way analyses of variance (ANOVAs) and Tukey’s HSD post-hoc tests were conducted to examine pairwise comparisons. *Abbreviations: AE, androstenedione; AH, anterior hypothalamus; PAG, periaqueductal gray; PROG, progesterone.* Symbols: NS (not significant, *p* > 0.10), #*p* < 0.10, \**p* < 0.05, \*\*\**p* < 0.001. *Outliers excluded from statistical analysis: one LD female for AE:PROG synthesis in the adrenal glands, two SD males for PROG synthesis in the PAG, one LD-M male for AE synthesis in the PAG, and one LD male, one LD-M male, and one LD-M female for AE:PROG synthesis in the PAG.*

**Table S10.** Summary ofunivariate statistical analyses performed to examine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on adrenal and neural 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase (3β-HSD) activity in male and female hamsters.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Tissue or Brain Region** | **Statistical Test and Family** | **Coefficients** | **Estimate ± SE** | **df** | ***F*** | ***p*** | ***\**** |
| **PROG Synthesis** | Adrenal Glands | Two-Way ANOVA | Treatment | 43.03 ± 40.89 | 2,38 | 0.255 | 0.776 | NS |
|  |  |  | Sex | 55.19 ± 184.52 | 1,38 | 8.585 | **0.006** | \*\* |
|  |  |  | Treatment\*Sex | -61.20 ± 57.83 | 2,38 | 1.495 | 0.237 | NS |
| **PROG Synthesis** | AH | Two-Way ANOVA | Treatment | 1.328 ± 0.574 | 2,36 | 2.968 | ***0.064*** | # |
|  |  |  | Sex | 0.006 ± 0.123 | 1,36 | 1.456 | 0.236 | NS |
|  |  |  | Treatment\*Sex | -0.371 ± 0.178 | 2,36 | 2.839 | ***0.072*** | # |
| **PROG Synthesis** | PAG | Two-Way ANOVA | Treatment | -0.013 ± 0.034 | 2,31 | 0.987 | 0.384 | NS |
|  |  |  | Sex | 0.206 ± 0.155 | 1,31 | 0.998 | 0.326 | NS |
|  |  |  | Treatment\*Sex | -0.050 ± 0.048 | 2,31 | 0.773 | 0.470 | NS |
| **AE Synthesis** | Adrenal Glands | Two-Way ANOVA | Treatment | 437.8 ± 272.5 | 2,38 | 0.607 | 0.550 | NS |
|  |  |  | Sex | 394.6 ± 1229.7 | 1,38 | 12.62 | **0.001** | \*\* |
|  |  |  | Treatment\*Sex | -482.0 ± 385.4 | 2,38 | 1.297 | 0.285 | NS |
| **AE Synthesis** | AH | Two-Way ANOVA | Treatment | -0.629 ± 0.726 | 2,38 | 3.175 | ***0.053*** | # |
|  |  |  | Sex | 1.674 ± 3.277 | 1,38 | 0.638 | 0.429 | NS |
|  |  |  | Treatment\*Sex | -0.749 ± 1.027 | 2,38 | 0.567 | 0.572 | NS |
| **AE Synthesis** | PAG | Two-Way ANOVA | Treatment | -0.122 ± 0.210 | 2,37 | 0.279 | 0.758 | NS |
|  |  |  | Sex | -0.028 ± 0.948 | 1,37 | 2.147 | 0.151 | NS |
|  |  |  | Treatment\*Sex | 0.125 ± 0.297 | 2,37 | 0.113 | 0.893 | NS |
| **AE:PROG Synthesis** | Adrenal Glands | Two-Way ANOVA | Treatment | -0.158 ± 2.002 | 2,37 | 0.771 | 0.470 | NS |
|  |  |  | Sex | 11.64 ± 6.389 | 1,37 | 3.337 | ***0.076*** | # |
|  |  |  | Treatment\*Sex | 1.146 ± 2.901 | 2,37 | 0.130 | 0.879 | NS |
| **AE:PROG Synthesis** | AH | Two-Way ANOVA | Treatment | -0.900 ± 2.898 | 2,35 | 0.124 | 0.884 | NS |
|  |  |  | Sex | -20.26 ± 14.10 | 1,35 | 5.080 | **0.031** | \* |
|  |  |  | Treatment\*Sex | 3.959 ± 4.336 | 2,35 | 0.519 | 0.600 | NS |
| **AE:PROG Synthesis** | PAG | Two-Way ANOVA | Treatment | -4.981 ± 6.494 | 2,28 | 0.223 | 0.801 | NS |
|  |  |  | Sex | 37.59 ± 21.54 | 1,28 | 3.059 | ***0.091*** | # |
|  |  |  | Treatment\*Sex | 4.654 ± 9.583 | 2,28 | 1.465 | 0.248 | NS |

Two-way analyses of variance (ANOVAs) were used to examine the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on adrenal and neural 3β-HSD activity in male and female hamsters (males – LD: *n* = 4-6, LD-M: *n* = 6-8, SD: *n* = 6-8; females – LD: *n* = 4-6, LD-M: *n* = 5-8, SD: *n* = 8). Coefficient estimates ± standard error (SE), degrees of freedom (df), *F*-statistics (*F*), *p*-values (*p*), and statistical significance (\*) for each model are shown. **Boldface** indicates *p* < 0.05, whereas **boldface** and *italics* indicate *p* < 0.10. *F*-statistics and estimations of effect size (*R*2) for each model are provided in the Results section of the main text. *Abbreviations: AE, androstenedione; AH, anterior hypothalamus; PAG, periaqueductal gray; PROG, progesterone.* Symbols: NS (not significant, *p* > 0.10), #*p* < 0.10, \**p* < 0.05, \*\**p* < 0.01. *Outliers excluded from statistical analysis: one LD female for AE:PROG synthesis in the adrenal glands, two SD males for PROG synthesis in the PAG, one LD-M male for AE synthesis in the PAG, and one LD male, one LD-M male, and one LD-M female for AE:PROG synthesis in the PAG.*

**Table S11.** Treatment with timed melatonin injections and short-day photoperiods had sex-specific effects on the ratio of androstenedione (AE) synthesis to progesterone (PROG) synthesis via 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase (3β-HSD) in the adrenal glands and brain of Siberian hamsters.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Tissue or Brain Region** | **Males** | | | **Females** | | |
|  | **LD** | **LD-M** | **SD** | **LD** | **LD-M** | **SD** |
| AE:PROG Synthesis | Adrenals | 10.32 ± 1.992a | 12.67 ± 3.948a | 10.26 ± 2.721a | 5.141 ± 1.211a | 10.12 ± 3.045**b** | 7.781 ± 1.417a,b |
| AE:PROG Synthesis | AH | 13.77 ± 3.664a | 16.39 ± 6.531a | 12.32 ± 4.095a | 2.681 ± 0.963a | 6.057 ± 1.337a,b | 8.873 ± 2.739**b** |
| AE:PROG Synthesis | PAG | 22.55 ± 10.90a | 29.43 ± 11.60a | 19.86 ± 7.247a | 4.449 ± 1.340a | 6.708 ± 1.485a,b | 19.76 ± 6.161**b** |

Means ± SEM (males – LD: *n* = 4-6, LD-M: *n* = 6-8, SD: *n* = 8; females – LD: *n* = 4-6, LD-M: *n* = 5-8, SD: *n* = 8) of the ratio of AE:PROG synthesis in the adrenal glands, anterior hypothalamus (AH), and periaqueductal gray (PAG) of long-day hamsters (LD), LD hamsters administered timed melatonin injections (LD-M), and short-day hamsters (SD) following 10 weeks of treatment. Different letters indicate a significant difference between treatment groups within each sex (*p* < 0.05).

**FIGURES**

Diagram

Description automatically generated

**Figure S1.** Time course of **(A, B)** progesterone (PROG) synthesis and **(C, D)** androstenedione (AE) synthesis by 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase (3β-HSD)in the adrenal glands **(A, C)** and brain **(B, D)** of Siberian hamsters.

Chart, waterfall chart

Description automatically generated

**Figure S2.** 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase (3β-HSD) assay validation using pharmacological inhibitors of steroidogenic enzymes. Trilostane, a competitive inhibitor of 3β-HSD, significantly reduced progesterone (PROG; **A, B**) and androstenedione (AE) production **(C, D)** in the adrenal glands **(A, C)** and brain **(B, D)** relative to control samples. Similarly, control samples, which included abiraterone acetate and fadrozole hydrochloride (pharmacological inhibitors of cytochrome P450c17 and aromatase, respectively), had significantly higher 3β-HSD activity than samples containing no inhibitors, indicating that these compounds reduced the metabolism of formed PROG-c2d2 and AE-d2. Bar heights represent means ± SEM (*n* = 3 per group), and “\*” indicates a significant difference relative to control samples (*p* < 0.05; Welch’s t-tests).

Diagram, engineering drawing

Description automatically generated

**Figure S3.** Quantification of progesterone production by 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase in Siberian hamster adrenal and brain tissue via liquid chromatography-tandem mass spectrometry (LC-MS/MS).Representative extracted ion chromatographs of fragment ions from **(A, B)** progesterone-c2d2, (insets, **A, B**) progesterone-d9, **(C, D)** progesterone, and **(E, F)** pregnenolone-c2d2 in the adrenal glands **(A, C, E)** and brain **(B, D, F)**. MS/MS transitions for precursor and fragment ions are provided in table S2 (electronic supplementary material).

Diagram, engineering drawing

Description automatically generated**Figure S4.** Quantification of androstenedione production by 3β-hydroxysteroid dehydrogenase/∆5-∆4 isomerase in Siberian hamster adrenal and brain tissue via liquid chromatography-tandem mass spectrometry (LC-MS/MS).Representative extracted ion chromatographs of fragment ions from **(A, B)** androstenedione-d2, (insets, **A, B**) androstenedione-d7, **(C, D)** androstenedione, and **(E, F)** dehydroepiandrosterone-d2 in the adrenal glands **(A, C, E)** and brain **(B, D, F)**. MS/MS transitions for precursor and fragment ions are provided in table S2 (electronic supplementary material).

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