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

Kathleen M. Munley[1] | Jonathan C. Trinidad[2] | Jessica E. Deyoe[1] | Catherine H. Adaniya[1] | Andrea M. Nowakowski[1] | Clarissa C. Ren[1] | Grace V. Murphy[1] | John M. Reinhart[1] | Gregory E. Demas[1]

[1]Department of Biology and Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN, USA

[2]Department of Chemistry, Indiana University, Bloomington, IN, USA

Correspondence
Kathleen M. Munley, Department of Biology and Center for the Integrative Study of Animal Behavior, Indiana University, 1001 East Third Street, Bloomington, IN 47405, USA.
Email: kmunley@indiana.edu

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Abstract

Aggression is a complex social behaviour that allows individuals to compete for access to limited resources (eg, mates, food and territories). Excessive or inappropriate aggression, however, has become problematic in modern societies, and current treatments are largely ineffective. Although previous work in mammals suggests that aggressive behaviour varies seasonally, seasonality is largely overlooked when developing clinical treatments for inappropriate aggression. Here, we investigated how the hormone melatonin regulates seasonal changes in neurosteroid levels and aggressive behaviour in Siberian hamsters, a rodent model of seasonal aggression. Specifically, we housed males in long-day (LD) or short-day (SD) photoperiods, administered timed s.c. melatonin injections (which mimic a SD-like signal) or control injections, and measured aggression using a resident-intruder paradigm after 9 weeks of treatment. Moreover, we quantified five steroid hormones in circulation and in brain regions associated with aggressive behaviour (lateral septum, anterior hypothalamus, medial amygdala and periaqueductal gray) using liquid chromatography-tandem mass spectrometry. SD hamsters and LD hamsters administered timed melatonin injections (LD-M) displayed increased aggression and exhibited region-specific decreases in neural dehydroepiandrosterone, testosterone and oestradiol, but showed no changes in progesterone or cortisol. Male hamsters also showed distinct associations between neurosteroids and aggressive behaviour, in which neural progesterone and dehydroepiandrosterone were positively correlated with aggression in all treatment groups, whereas neural testosterone, oestradiol and cortisol were negatively correlated with aggression only in LD-M and SD hamsters. Collectively, these results provide insight into a novel neuroendocrine mechanism of mammalian aggression, in which melatonin reduces neurosteroid levels and elevates aggressive behaviour.

KEYWORDS
aggression, LC-MS/MS, neurosteroids, pineal, seasonality, social behaviour network

1 | INTRODUCTION

Aggression is a well-studied behaviour that has evolved to allow individuals to compete for limited resources in their environment, such as food, territories and mates.[1,2] While high levels of aggressive behaviour can be advantageous, particularly for wild mammals; excessive or inappropriate aggression in humans (eg, aggression that is exaggerated, persistent and/or expressed out of context) is problematic and may be associated with a suite of neurological and psychiatric disorders, including mood disorders, anxiety and psychotic disorders, and impulse control and conduct disorders.[3-6]; but see also [7,8] Despite the pervasiveness of inappropriate aggression in modern societies, current pharmacological treatments are largely ineffective. Antipsychotics and neuroleptics are the most commonly used drugs to treat excessive or inappropriate aggression, although many of these medications are non-specific and often result in unwanted side effects, especially during chronic treatment.[9-11] Thus, to develop better treatments for inappropriate aggression, it is necessary to have a comprehensive understanding of the molecules and neural circuits that modulate aggressive behaviour, which can only be accomplished through the use of animal models.

Interestingly, while it is well-established that aggression changes seasonally in mammals, including humans, seasonality is often overlooked when developing clinical treatments for inappropriate aggression. In the wild, aggressive behaviour varies seasonally in mammals based on environmental resources and/or the timing of reproduction.[1,2,12] There is evidence that aggression also varies seasonally in humans, particularly in temperate regions that undergo profound changes in photoperiod (ie, day length).[13,14] In mammals, photoperiod is translated into a biochemical signal via a complex neural circuit, in which environmental light is perceived by retinal ganglion cells, processed in the hypothalamus and transduced from a neural to an endocrine signal through the release of melatonin by the pineal gland.[15,16] Because melatonin secretion is high at night and low during the day, the pattern and duration of melatonin secretion conveys information about day length to the central nervous system and, thus, is important in establishing and maintaining biological rhythms.[17,18] Although recent studies suggest that human aggression may be modulated by melatonin,[19,20] the precise mechanisms that regulate seasonal variation in aggressive behaviour are unknown.

Siberian hamsters (*Phodopus sungorus*) are an excellent animal model for studying the neuroendocrine circuits underlying seasonal aggression because this species shows high levels of aggression year-round and primarily uses photoperiod as an environmental cue to coordinate seasonal changes in aggressive behaviour and its underlying physiological mechanisms.[21,22] Previously, we have shown that Siberian hamsters housed in short-day photoperiods (SDs, characteristic of non-breeding season) undergo gonadal regression and decrease circulating gonadal steroid levels (eg, testosterone [T] and 17β-oestradiol [E_2]) but display increased aggression.[23-26] Interestingly, elevated aggression during SDs is associated with increases in circulating levels of the adrenal androgen dehydroepiandrosterone (DHEA),[27,28] suggesting that adrenal androgens may be important in regulating non-breeding aggression in this species. Moreover, there is some evidence that melatonin regulates this neuroendocrine circuit. Male and female hamsters housed in long-day photoperiods and given timed melatonin injections, which mimic SD-like patterns of melatonin secretion, exhibit increased aggression.[26-28] We have also found that short-term exogenous melatonin elevates aggressive behaviour in LD males, yet causes no change in gonadal steroid levels[28]; whereas long-term melatonin treatment increases aggression, induces gonadal regression and causes SD-like changes in circulating androgen levels in these animals.[26] In addition, treating adrenal glands with melatonin in vitro elevates DHEA output in SD, but not LD females, whereas treating cultured ovaries with melatonin elevates DHEA output in LD, but not SD females.[27] These data suggest that melatonin modulates seasonal changes in peripheral steroidogenesis and aggressive behaviour; however, it is not known whether melatonin may mediate seasonal aggression by altering steroid synthesis and metabolism in the brain.

While few studies have examined seasonal changes in neural steroid signalling in Siberian hamsters, changes in steroid synthesis, metabolism and receptor abundance have been reported in other seasonally breeding species. Many of these changes occur in nodes of the social behaviour network, a collection of midbrain, hypothalamic and basal forebrain nuclei that are sensitive to steroid hormones and have been implicated in the regulation of social behaviour in vertebrates,[29,30] including sexual behaviour,[31-33] parental care[34,35] and aggressive behaviour.[9,36,37] In mammals, these nodes include the extended medial amygdala (ie, the medial amygdala and the bed nucleus of the stria terminalis), lateral septum, preoptic area, anterior hypothalamus, ventromedial hypothalamus and periaqueductal gray.[29,38] For example, seasonal changes in neurosteroid synthesis and signalling in the brain, including increased expression of steroidogenic enzymes (eg, 3β-hydroxysteroid dehydrogenase and CYP19) and steroid receptors (eg, androgen and oestrogen receptors), have been implicated in regulating aggressive behaviour in song sparrows (*Melospiza melodia*),[39-43] beach mice (*Peromyscus polionotus*) and deer mice (*Peromyscus maniculatus*).[44,45] Furthermore, SD male and female Siberian hamsters display increases in oestrogen receptor α abundance in brain regions associated with aggressive, but not reproductive behaviours,[46,47] suggesting that local changes in steroid production and signalling mechanisms in the brain may enable seasonally breeding animals to maintain or increase aggressive behaviour, despite low levels of circulating gonadal steroids.

The present study aimed to assess the role of melatonin in regulating neurosteroid levels and aggressive behaviour in male Siberian hamsters. Adult male hamsters were housed in LDs or SDs, and a subset of LD animals were administered timed melatonin injections, which summated with the endogenous melatonin profile of these animals to mimic a SD-like signal. Following 9 weeks of treatment, aggressive and non-aggressive social behaviours were measured and five steroid hormones (progesterone [PROG], DHEA, T, E_2 and

cortisol [CORT]) were quantified in circulation and in four brain regions that have been implicated in aggressive behaviour (the lateral septum, anterior hypothalamus, medial amygdala and periaqueductal gray) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We hypothesised that melatonin facilitates increased aggression during the non-breeding season by elevating the conversion of circulating and neurally derived prohormones (ie, PROG and DHEA) to biologically active steroids (ie, E_2 and CORT). Thus, we predicted that male hamsters given a SD-like melatonin signal, either via timed melatonin injections or exposure to SDs, will exhibit gonadal regression, a reduction in body mass, and decreases in PROG, DHEA and T concentrations in circulation and in the brain, but will display increases in circulating and neural E_2 and CORT levels and aggressive behaviour.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Adult male Siberian hamsters (*P. sungorus*, > 60 days of age) were reared and maintained in a breeding colony under long days (light:dark, 16:8 hours; lights off at 4.00 PM Eastern Standard Time, EST) and group-housed at weaning (post-natal day 18) in polypropylene cages (28 × 17 × 12 cm). Sani-chip bedding (Teklad, laboratory grade; Envigo, Indianapolis, IN, USA) was used in each cage and hamsters were given ad libitum access standard laboratory rodent chow (Teklad global 18% protein diet; Envigo) and tap water. Ambient temperature was maintained at 20 ± 2°C and relative humidity was maintained at 55 ± 5%. All procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) at Indiana University (protocol #17-001).

2.2 | Photoperiodic manipulations and in vivo melatonin administration

Prior to the start of photoperiodic manipulations, experimental male hamsters (n = 45) were individually housed for a 2-week acclimation period on a LD light cycle. Following acclimation, animals were transferred to a room on a SD light cycle (n = 23; light:dark, 8:16 hours; lights on at 08:00 AM EST) or were relocated to a new room on a LD light cycle (n = 22). Melatonin profiles were manipulated in a subset of LD hamsters (LD-M, n = 13), which were administered timed s.c. injections of melatonin (15 μg day^{-1}; [M5250; Sigma-Aldrich, St Louis, MO, USA] dissolved in 1:10 ethanol:saline solution), as described previously.[26,27,48] All remaining animals in the study (n = 32) received daily injections of a control (1:10 ethanol:saline) solution. Injections were administered 2 hours prior to lights out (1.30 PM to 2.30 PM EST), which extended the LD pattern of endogenous melatonin secretion to mimic that of SD animals.[48] Hamsters remained in photoperiodic treatments and were administered melatonin or control injections for 9 weeks to capture seasonal and melatonin-dependent changes in body mass, reproductive physiology, social behaviour, and circulating and neural steroid levels.

2.3 | Seasonal phenotypes

Following 9 weeks of treatment, seasonal phenotypes were determined based on a priori criteria that have been previously described for this species.[24,25] Body mass was measured weekly for the duration of the study (see Supporting information, Figure S1) and paired testes were collected and weighed at the end of the study. These two measures, which have been reported previously,[26] were the primary criteria used to classify animals as responsive or non-responsive to photoperiodic treatment. Coat colour was also assessed via visual inspection at the end of the study and was used to help confirm each classification. For all animals in the LD and SD groups, each of the three variables used for classification (body mass, paired testes mass and coat colour) were in agreement. LD hamsters had functional testes (ie, had a paired testes mass between 0.600 and 0.900 g), displayed no significant change in body mass (< 4%) and had brown-grey pelage. By contrast, SD animals that were responsive to photoperiodic treatment (n = 16) had regressed testes (ie, had a paired testes mass that was > 2 standard deviations below the mean paired testes mass of LD animals), showed a significant change in body mass (≥ 4%) and had white pelage on the chest, lateral flanks and face. LD-M hamsters exhibited a phenotype that was intermediate to LD and SD hamsters. These animals had regressed testes, showed no significant change in body mass, and had white pelage on the chest and face, but not the lateral flanks. A subset of SD animals (n = 7; 30.4%) failed to respond to photoperiodic treatment and were classified as "non-responders" using the same criteria described above for LD animals. These animals were excluded from the study as a result of insufficient numbers for statistical analysis. Non-responsiveness to SDs, in which animals do not undergo gonadal regression or reduce body mass in response to SDs and generally respond physiologically and behaviourally like LD animals, has been previously documented in this species and affects between 10% and 50% of the population.[46,49-51]

2.4 | Behavioural testing

After 9 weeks of treatment, territorial aggression was quantified within the first 3 hours of the dark phase (4.30 PM to 7.30 PM EST) using a 5-minute same-sex resident-intruder paradigm, as described previously (see Supporting information, Appendix S1).[26,27] Aggressive (ie, latency to first attack, number and duration of attacks, and number and duration of chases) and non-aggressive social behaviours (ie, number and duration of nose-to-nose investigations, anogenital investigations, scent marking events, and

self-grooming events) were scored for each experimental animal. Measures of aggression, investigation, scent marking and grooming were curated from a previously reported study[26] and were defined according to prior studies on same-sex aggression in male Siberian hamsters.[25,26,52]

2.5 | Blood sampling and tissue collection

To assess seasonal changes in circulating and neural steroid levels, a terminal blood sample and brain tissues were collected from each experimental animal following behavioural testing (4.40 PM to 7.40 PM EST). Animals were lightly anaesthetised using isoflurane (Isothesia; Henry Schein Animal Health, Melville, NY, USA) and blood was drawn from the retro-orbital sinus into microcapillary tubes. Blood samples were collected into polypropylene microcentrifuge tubes within 2 minutes following behavioural testing, flash frozen on dry ice and stored at −20°C until further analysis. Immediately following the collection of blood samples (≤ 5 minutes following behavioural testing), animals were killed with a lethal i.p. injection (0.3 mL) of ketamine (150 mg kg^{-1}) and xylazine (30 mg kg^{-1}) cocktail in 0.9% saline. Whole brains were rapidly extracted (≤ 5 minutes following euthanasia), flash frozen on crushed dry ice and stored at −80°C until processing. Paired testes and epididymal white adipose tissue (EWAT) were also removed and weighed individually to the nearest mg. Data for paired testes mass and EWAT mass were utilised from a previously reported study.[26]

2.6 | Tissue processing

Brains were covered in Tissue-Tek® OCT compound (Sakura, Osaka, Japan) and coronal sections (thickness: 300 μm) were cut on a sliding freezing microtome (Rankin Biomedical, Holly, MI, USA) and mounted on microscope slides. Frozen brain regions of interest were microdissected with Miltex™ disposable biopsy punches (1 mm diameter, 0.245 mg per punch; Integra LifeSciences, Plainsboro Township, NJ, USA) using the Palkovits punch technique.[53] Microdissected punches for steroid analysis were collected from both hemispheres for each experimental animal. Locations of specific brain regions were determined using major anatomical landmarks and a mouse brain atlas.[54] Brain regions of interest included the lateral septum (LS), anterior hypothalamus (AH), medial amygdala (MeA) and periaqueductal gray (PAG). Microdissected brain punches of the LS (wet weight: 3.26 ± 0.08 mg), AH (1.86 ± 0.07 mg), MeA (2.78 ± 0.07 mg) and PAG (2.74 ± 0.08 mg) and 3 μL of blood from each animal were placed into 2-mL polypropylene microtubes (Sarstedt Inc., Newton, NC, USA) containing five zirconium ceramic beads (1.4 mm diameter; Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80°C until steroid extraction. Brain sections were Nissl stained with cresyl violet and imaged using a Motic EasyScan digital slide scanner (Kowloon, Hong Kong) to confirm the locations of microdissected punches.

2.7 | Steroid extraction for LC-MS/MS

Prior to homogenisation, isotopically labelled analogues of steroid hormones of interest were added to each sample, which were used to quantify endogenous steroid concentrations. The following isotopically labelled compounds and amounts were used: 100 pg cortisol-d_4, 200 pg oestradiol-d_4, 100 pg testosterone-c_3, 100 pg dehydroepiandrosterone-d_6 and 100 pg progesterone-d_9 in 20% acetonitrile with 0.1% formic acid. In addition, 5 ng pregnanediol in 20% acetonitrile with 0.1% formic acid was spiked into each sample to alleviate the stickiness of isotopically labelled and endogenous steroids for plastics and glass used in the steroid extraction procedure. A blank sample, which contained 200 μL of 100% acetonitrile with 0.1% formic acid (homogenisation solution) spiked with 100 pg cortisol-d_4, 200 pg oestradiol-d_4, 100 pg testosterone-c_3, 100 pg dehydroepiandrosterone-d_6, 100 pg progesterone-d_9 and 5 ng pregnanediol, was processed alongside blood and brain tissue samples and was used to track recovery after steroid extraction. All samples were homogenised in 100% acetonitrile with 0.1% formic acid using a bead mill homogeniser (Bead Ruptor 24 Elite; Omni International, Kennesaw, GA, USA), incubated for 1 hour at −20°C, centrifuged at room temperature, dried using an Evap-O-Rac system (Cole-Parmer, Vernon Hills, IL, USA) and reconstituted in 20% methanol with 0.1% formic acid (see Supporting information, Appendix S1).

Steroids were extracted from reconstituted blood and brain tissue samples using an acetonitrile-based solid phase extraction protocol with C18 OMIX tips (Agilent Technologies, Santa Clara, CA, USA) (see Supporting information, Appendix S1). In addition to blood and brain tissue extracts, 5 μL of isotopically labelled steroids, which contained 100 pg cortisol-d_4, 200 pg oestradiol-d_4, 100 pg testosterone-c_3, 100 pg dehydroepiandrosterone-d_6 and 100 pg progesterone-d_9 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 steroids, was run alongside blood and brain tissue samples and was used to track recovery for LC-MS/MS. All samples were dried in a CentriVap vacuum concentrator (Labconco, Kansas City, MO, USA), reconstituted in 15 μL 20% acetonitrile with 0.1% formic acid, sealed with crimp top caps (11 mm diameter; Agilent Technologies) and stored at −20°C until LC-MS/MS analysis.

2.8 | LC-MS/MS analysis

Concentrations of PROG, DHEA, T, E_2 and CORT and their corresponding isotopically labelled analogues (progesterone-d_9, dehydroepiandrosterone-d_6, testosterone-c_3, oestradiol-d_4 and cortisol-d_4, respectively) were measured in blood and brain tissue extracts using an Easy NanoLC 1200 HPLC coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) (see Supporting information, Appendix S1). MS/MS transitions for endogenous and isotopically labelled steroids, including retention times and mass-to-charge ratios of precursor and fragment ions, are

available in the Supporting information (Table S1). LC-MS/MS data were analysed using the XCALIBUR QUAN BROWSER, version 2.2 (Thermo Fisher Scientific). PROG, DHEA, T, E_2 and CORT concentrations in blood and brain tissue samples were calculated using isotopic internal standard quantification, in which the amount of the isotopically labelled analogue of each steroid was multiplied by the ratio of the fragment ion signal for the endogenous steroid to the fragment ion signal for the isotopically labelled analogue.[55,56] Percentage recoveries of isotopically labelled steroids after steroid extraction and accuracy and precision measurements of isotopically labelled steroids for the LC-MS/MS protocol are provided in the Supporting information (Tables S2 and S3). The E_2 concentration of 11 blood (52.4% of samples; LD: n = 3, LD-M: n = 4, SD: n = 4) and 51 brain samples (58.0% of samples; LD: n = 12, LM: n = 18, SD: n = 21) was below the limit of detection for the assay. These values were considered non-detectable and the E_2 content for these samples was set to the limit of detection (1 ng) for the purpose of statistical analysis.

2.9 | Statistical analysis

All data are presented as the mean ± SEM. Statistical testing was performed using R, version 4.0.2.[57] $P < 0.05$ was considered statistically significant after controlling for the false discovery rate.[58] Statistical outliers were examined using Grubbs' tests,[59] and data points that affected the conceptual conclusions of the study were excluded from statistical analysis. One-way multivariate ANOVA (MANOVA) was used to assess the effects of photoperiodic treatment and melatonin administration on body mass, investigation, self-grooming, and neural DHEA and CORT levels.[57] Non-parametric permutational MANOVA (PERMANOVA) with 999 permutations was used to examine the effects of photoperiodic and melatonin treatment on reproductive physiology, aggression, scent marking, circulating steroid levels, and neural PROG, T and E_2 levels based on Euclidean distances (see Supporting information, Tables S4 and S5).[60] If a multivariate test reported a significant effect of treatment ($P < 0.05$) or a trend towards an effect of treatment ($P < 0.10$) for one or more of the dependent variables included in a given analysis, univariate one-way ANOVA[57] or non-parametric Kruskal-Wallis one-way ANOVA on ranks[57] and post-hoc testing (Tukey's honestly significant difference [HSD] tests for one-way ANOVA[61] and Dunn's tests for multiple comparisons for Kruskal-Wallis one-way ANOVA on ranks[62]) were conducted to examine pairwise comparisons. Spearman's rank correlations with a Holm-Bonferroni correction for multiple comparisons were computed to determine whether associations between aggressive behaviour, circulating steroid levels and neurosteroid levels differed by treatment group,[63] and a PERMANOVA with 999 permutations was used to determine whether relationships between aggression and steroid concentrations were affected by photoperiodic and melatonin treatment based on Euclidean distances (see Supporting information, Appendix S1).[60] Effect sizes were calculated for all multivariate and univariate analyses and their respective post-hoc tests and are expressed as partial η^2 for one-way MANOVA, R^2 for PERMANOVA, η^2 for one-way ANOVA, transformed η^2 for Kruskal-Wallis one-way ANOVA on ranks,[64,65] Cohen's *d* for Tukey's HSD post-hoc tests and Hedge's *g* for Dunn's post-hoc tests.[66] For η^2 and R^2 values, 0.04-0.25 indicates a small effect, 0.25-0.64 indicates a moderate effect and > 0.64 indicates a strong effect. For Cohen's *d* and Hedge's *g* values, |0.41-1.15| represents a small effect, |1.15-2.70| represents a moderate effect and |>2.70| represents a strong effect.[67]

3 | RESULTS

3.1 | Melatonin administration and exposure to SD photoperiods induced characteristic changes in reproductive physiology

Timed melatonin injections and exposure to SDs caused seasonally appropriate changes in reproductive physiology, including gonadal regression and a reduction in EWAT mass (Table 1). There was a significant effect of treatment on reproductive tissue mass (PERMANOVA: $F_{1,36} = 163.0$, $P < 0.001$, $R^2 = 0.819$) and body mass (one-way MANOVA: $F_{2,35} = 5.447$, $P < 0.001$, partial $\eta^2 = 0.237$) (see Supporting information, Table S4). Specifically, LD hamsters given timed melatonin injections (LD-M) and hamsters that were exposed to short-day photoperiods (SD) exhibited significant reductions in paired testes mass (Kruskal-Wallis one-way ANOVA on ranks: $H = 30.56$, $df = 2$, $P < 0.001$, $\eta^2 = 0.816$) and EWAT mass (Kruskal-Wallis one-way ANOVA on ranks: $H = 15.01$, $df = 2$, $P < 0.001$, $\eta^2 = 0.372$) compared to LD hamsters. These reductions in reproductive tissue mass, however, were more pronounced in SD hamsters than in LD-M hamsters (paired testes mass: $P < 0.001$, $g = -2.013$; EWAT mass: $P = 0.022$, $g = -0.979$) (Table 1). SD hamsters also had a significantly lower body mass (one-way ANOVA: $F_{2,35} = 5.901$, $P = 0.006$, $\eta^2 = 0.252$) and percentage change in body mass (one-way ANOVA: $F_{2,35} = 15.60$, $P < 0.001$, $\eta^2 = 0.471$) than LD hamsters. There were no significant differences in body mass or percentage change in body mass, however, between LD and LD-M hamsters (body mass: $P = 0.663$, $d = -0.433$; percentage change in body mass: $P = 0.316$, $d = -0.735$) (Table 1; see also Supporting information, Figure S1).

3.2 | Treatment with melatonin or SD photoperiods increased aggressive, but not non-aggressive social behaviours

LD-M and SD animals displayed increased aggression relative to LD animals (Figure 1). Specifically, there was a trend towards an effect of treatment on aggressive behaviour (PERMANOVA: $F_{1,36} = 3.260$, $P = 0.075$, $R^2 = 0.083$) (see Supporting information, Table S4). LD-M animals had a longer attack duration than LD animals (Kruskal-Wallis one-way ANOVA on ranks: $H = 5.557$, $df = 2$, $P = 0.009$, $g = 0.800$), SD animals trended towards increases in number of attacks and attack

TABLE 1 Melatonin administration reduced reproductive tissue mass, but not body mass in male hamsters

	LD	LD-M	SD	*F* or *H* statistic	*df*	*P*
Body mass (g)	48.3 ± 1.84^{a}	45.9 ± 1.55^{a}	39.9 ± 1.84^{b}	5.901	2,35	**0.006**
Percentage change in body mass	1.22 ± 2.83^{a}	-4.74 ± 2.18^{a}	-17.8 ± 2.65^{b}	15.60	2,35	**< 0.001**
Paired testes mass (g)	0.71 ± 0.03^{a}	0.26 ± 0.04^{b}	0.06 ± 0.01^{c}	30.56	2	**< 0.001**
EWAT mass (g)	1.74 ± 0.12^{a}	1.31 ± 0.11^{b}	0.88 ± 0.12^{c}	15.01	2	**< 0.001**

Note: Mean ± SEM (LD: n = 9, LD-M: n = 13, SD: n = 16) of body mass, percentage change in body mass, paired testes mass and epididymal white adipose tissue (EWAT) mass in long-day animals (LD), LD animals administered timed melatonin injections (LD-M) and animals that were exposed to short-day photoperiods (SD) following 9 weeks of treatment. Significant *P* values are shown in bold and different superscript letters indicate a significant difference between treatment groups ($P < 0.05$; body mass and percentage change in body mass: one-way ANOVAs with Tukey's honestly significant difference post-hoc tests; paired testes mass and EWAT mass: Kruskal-Wallis one-way ANOVAs on ranks with Dunn's post-hoc tests for multiple comparisons).

duration relative to LD animals (number of attacks [Kruskal-Wallis one-way ANOVA on ranks]: $H = 2.421$, $df = 2$, $P = 0.061$, $g = 0.700$; attack duration: $P = 0.066$, $g = 0.546$) (Figure 1A) and both LD-M and SD animals had a significantly shorter attack latency than LD animals (LD-M [Kruskal-Wallis one-way ANOVA on ranks]: $H = 4.549$, $df = 2$, $P = 0.026$, $g = -0.811$; SD: $P = 0.030$, $g = -0.785$) (Figure 1B). In addition, SD animals showed a higher frequency and duration of chasing behaviour than LD animals (number of chases [Kruskal-Wallis one-way ANOVA on ranks]: $H = 3.245$, $df = 2$, $P = 0.037$, $g = 0.692$; chase duration [Kruskal-Wallis one-way ANOVA on ranks]: $H = 3.155$, $df = 2$, $P = 0.041$, $g = 0.675$) and LD-M animals trended towards an increase in chasing behaviour relative to LD animals (number of chases: $P = 0.097$, $g = 0.647$; chase duration: $P = 0.090$, $g = 0.662$) (Figure 1C,D). There was also a significant effect of treatment on scent marking behaviour (PERMANOVA: $F_{1,36} = 16.05$, $P < 0.001$, $R^2 = 0.308$) (see Supporting information, Table S4). SD animals displayed a higher frequency and duration of scent marking behaviour relative to LD animals (scent marking frequency [Kruskal-Wallis one-way ANOVA on ranks]: $H = 3.788$, $df = 2$, $P = 0.038$, $g = 0.615$; scent marking duration [Kruskal-Wallis one-way ANOVA on ranks]: $H = 3.548$, $df = 2$, $P = 0.040$, $g = 0.517$), whereas LD and LD-M animals showed no significant difference in scent marking behaviour (scent marking frequency: $P = 0.317$, $g = 0.358$; scent marking duration: $P = 0.303$, $g = 0.357$) (Figure 1E,F). There was no effect of treatment, however, on non-aggressive social behaviours, including self-grooming (one-way MANOVA: $F_{2,35} = 0.499$, $P = 0.736$, partial $\eta^2 = 0.028$) (Figure 1G,H) and investigation (one-way MANOVA: $F_{2,32} = 0.860$, $P = 0.555$, partial $\eta^2 = 0.103$) (see Supporting information, Table S4).

3.3 | Quantification of steroid hormones in hamster blood and brain tissue via LC-MS/MS

The steroid extraction and LC-MS/MS protocols developed in the present study allowed us to precisely and accurately measure five hormones within the sex steroid synthesis pathway using minute amounts of blood (3 μL) and tissue from discrete brain nuclei (0.98-4.41 mg) from male Siberian hamsters (Figure 2). Our highly sensitive LC-MS/MS methodology enabled us to consistently quantify concentrations of CORT (Figure 2A,B), T (Figure 2E,F), DHEA (Figure 2G,H) and PROG (Figure 2I,J), as well as their isotopically labelled analogues (cortisol-d_4, testosterone-c_3, dehydroepiandrosterone-d_6 and progesterone-d_9, respectively) in blood and brain tissue using isotopic internal standard quantification. E_2, however, was frequently non-detectable in blood and brain tissue, and E_2 levels that were detectable were often above the limit of detection but below the limit of quantification for our LC-MS/MS protocol (Figure 2C,D). Specifically, the E_2 concentration of 52.4% of blood samples (LD: n = 3, LD-M: n = 4, SD: n = 4) and 58.0% of brain tissue samples (LD: n = 12, LD-M: n = 18, SD: n = 21) was below the limit of detection, and the distribution of samples with non-detectable E_2 levels was similar among treatment groups.

To evaluate the accuracy and precision of our steroid extraction and LC-MS/MS protocols, percentage recoveries of isotopically labelled steroids were calculated following steroid extraction, and coefficients of variability and percentage recoveries of isotopically labelled steroids were measured for LC-MS/MS analysis (see Supporting information, Tables S2 and S3). The percentage recoveries after steroid extraction were 72.2%-117% in blood and 64.2%-110% in brain tissue, whereas the percentage recoveries for LC-MS/MS analysis were 2.28%-35.1% in blood and 7.71%-23.6% in brain tissue (see Supporting information, Tables S2 and S3). The coefficients of variability for the LC-MS/MS protocol were ≤ 25.8% in blood and ≤ 28.5% in brain tissue (see Supporting information, Table S3). These percentage recoveries and coefficients of variability are similar to values previously reported for solid phase extraction and LC-MS/MS protocols for steroid hormone quantification in rodents.[68-71]

3.4 | Circulating testosterone levels differed across seasonal phenotypes and in response to timed melatonin injections

There was a trend towards an effect of treatment on circulating steroid levels (PERMANOVA: $F_{1,19} = 2.872$, $P = 0.091$, $R^2 = 0.131$)

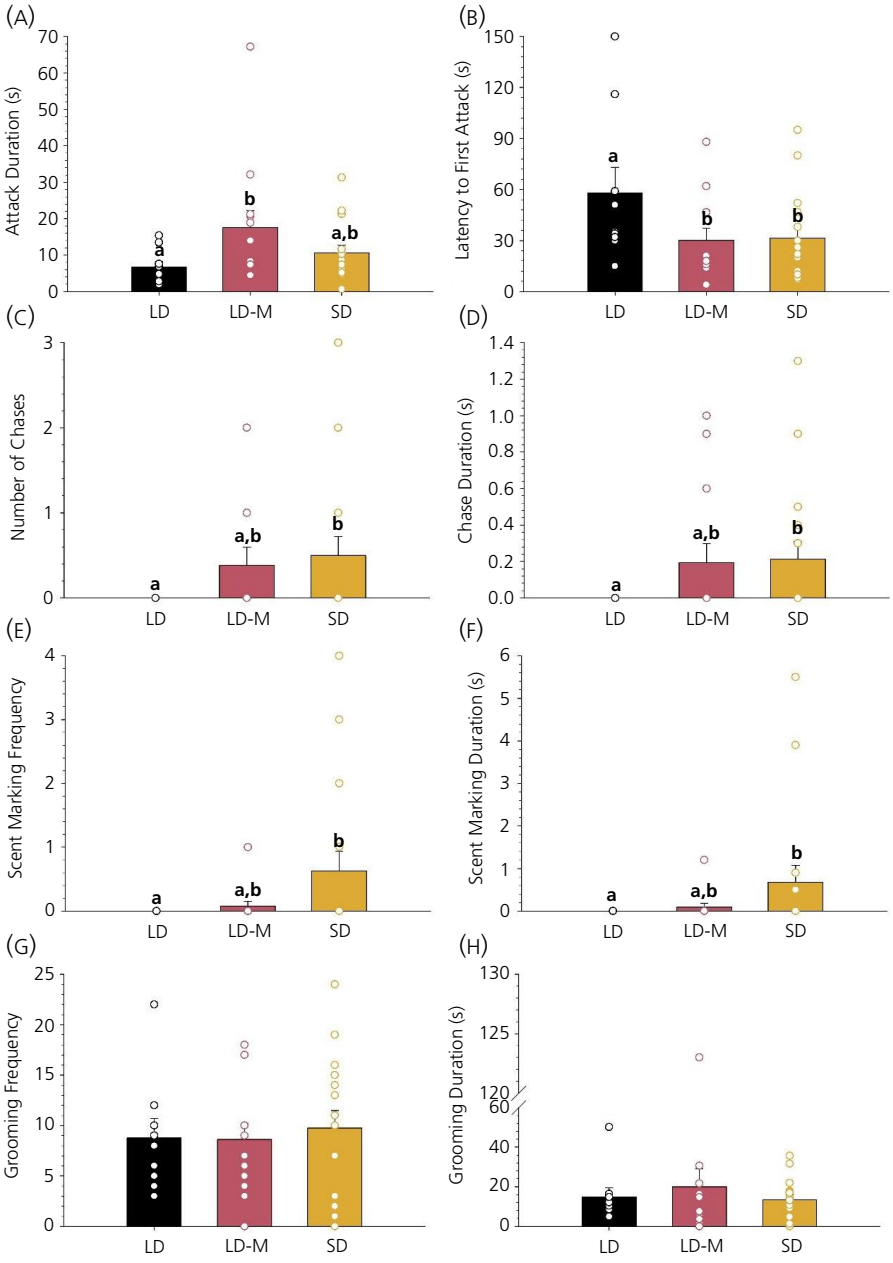

FIGURE 1 Timed melatonin administration and exposure to short-day photoperiods increased aggressive and scent marking behaviours, but did not affect self-grooming. (A) Attack duration, (B) latency to first attack, (C) number of chases, (D) chase duration, (E) scent marking frequency, (F) scent marking duration, (G) grooming frequency and (H) grooming duration of long-day hamsters (LD; black), LD hamsters given timed melatonin injections (LD-M; pink) and hamsters that were exposed to short-day photoperiods (SD; yellow). Data are presented as the mean ± SEM (LD: n = 9, LD-M: n = 12 or 13, SD: n = 15 or 16). Different lowercase letters indicate a significant difference between treatment groups ($P < 0.05$, Kruskal-Wallis one-way ANOVAs on ranks with Dunn's post-hoc tests for multiple comparisons). *Outliers excluded from statistical analysis (not shown): one LD-M animal and one SD animal for attack duration*

(see Supporting information, Table S5). SD animals had significantly lower levels of circulating T than LD animals (Kruskal-Wallis one-way ANOVA on ranks: $H = 5.742$, $df = 2$, $P = 0.009$, $g = -1.789$) and LD-M animals trended towards a decrease in circulating T relative to LD animals ($P = 0.054$, $g = -1.559$) (Figure 3C). SD animals also trended towards a decrease in circulating DHEA compared to LD animals (Kruskal-Wallis one-way ANOVA on ranks: $H = 2.113$, $df = 2$, $P = 0.080$, $g = -0.944$). There was no significant difference in circulating DHEA, however, between LD and LD-M animals ($P = 0.320$, $g = -0.210$) (Figure 3B). Moreover, there were no significant differences in circulating PROG (one-way ANOVA: $F_{2,18} = 0.240$, $P = 0.789$, $\eta^2 = 0.026$) (Figure 3A), E_2 (Kruskal-Wallis one-way ANOVA on ranks: $H = 0.599$, $df = 2$, $P = 0.741$, $\eta^2 = 0.078$; Figure 3D) or CORT (one-way ANOVA: $F_{2,15} = 0.618$, $P = 0.552$, $\eta^2 = 0.076$) (Figure 3E) between treatment groups.

3.5 | Melatonin administration and exposure to SDs reduced neurosteroid levels in a region-specific manner

Treatment with timed melatonin injections or SDs decreased neurosteroid concentrations in some, but not all brain regions of interest (Figure 4). Overall, there were few differences in neural prohormone levels between treatment groups (Figure 4B,C). Although there was no overall effect of treatment on neural DHEA levels (one-way MANOVA: $F_{2,16} = 1.067$, $P = 0.413$, partial $\eta^2 = 0.234$), there was a trend towards an effect of treatment on DHEA levels in the PAG (one-way MANOVA: $F_{2,16} = 2.848$, $P = 0.094$) (see Supporting information, Table S5). Specifically, SD hamsters had a lower DHEA concentration in the PAG than LD hamsters (Kruskal-Wallis one-way ANOVA on ranks: $H = 3.685$, $df = 2$, $P = 0.028$, $g = -1.308$),

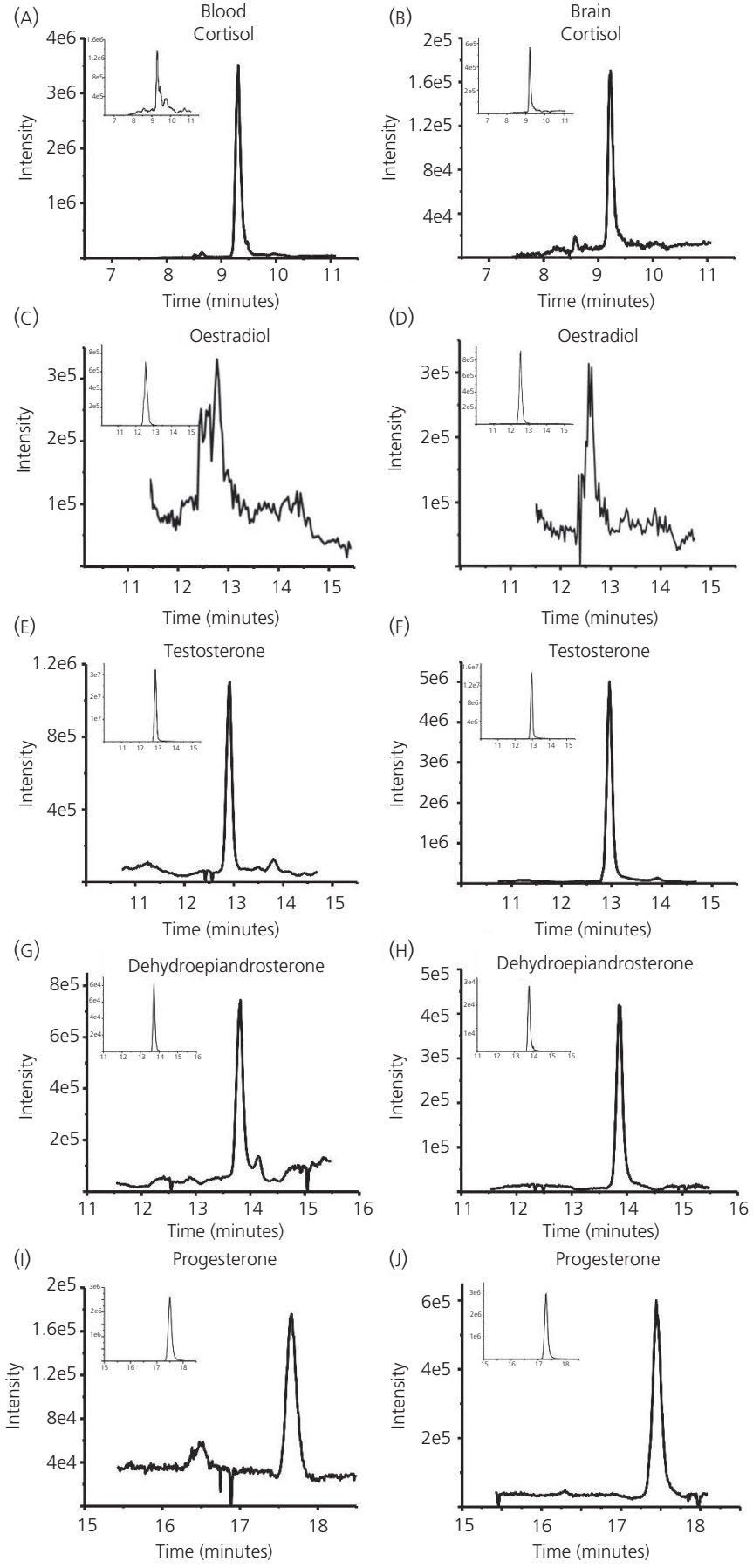

FIGURE 2 Quantification of steroid hormones in Siberian hamster blood and brain tissue via liquid chromatography-tandem mass spectrometry (**LC-MS/MS**). Representative extracted ion chromatographs of fragment ions from (A, B) cortisol, (C, D) oestradiol, (E, F) testosterone, (G, H) dehydroepiandrosterone and (I, J) progesterone and their corresponding isotopically labelled analogues (insets) in blood (A, C, E, G, I) and brain tissue (B, D, F, H, J) from male Siberian hamsters. MS/MS transitions for precursors and fragment ions are provided in the Supporting information (Table S1). Isotopically labelled analogues were spiked into samples prior to steroid extraction, and the ratio of the fragment ion signals for endogenous and isotopically labelled versions of each steroid was used to determine the concentrations of endogenous steroids. Although oestradiol was frequently observed above the level of detection, the signal-to-noise ratio of this steroid was below the level of quantification

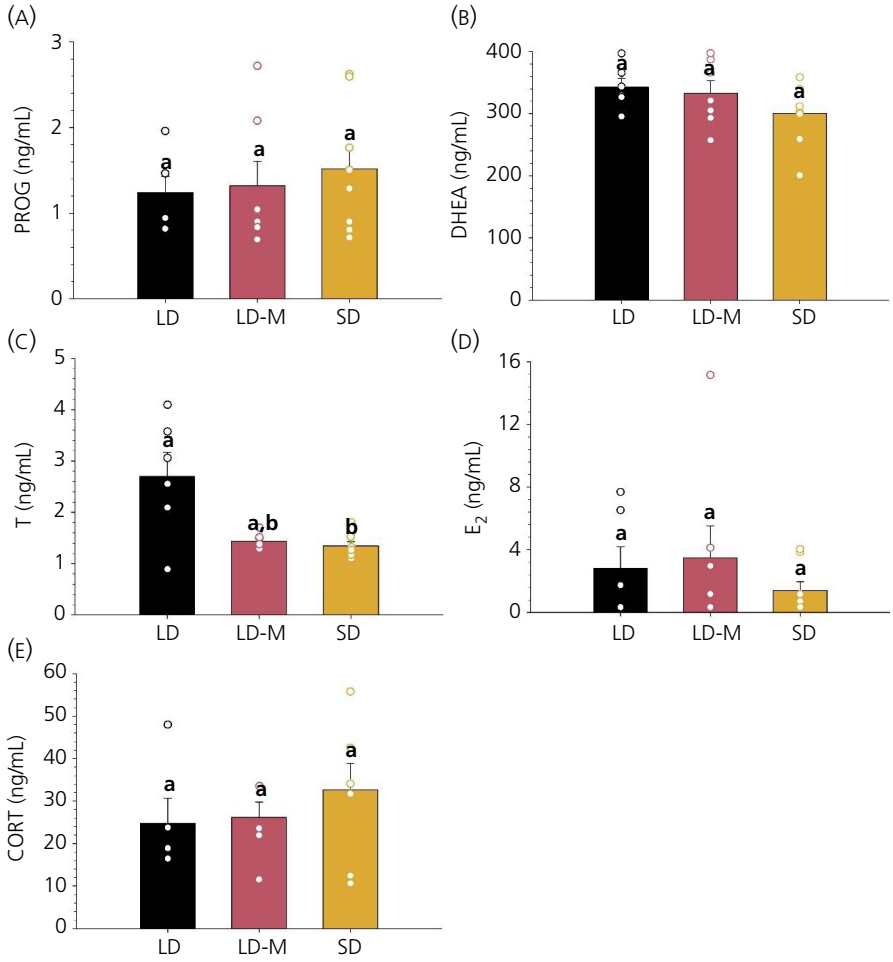

FIGURE 3 Male hamsters treated with timed melatonin injections or exposed to short days displayed reductions in circulating testosterone levels. Blood concentrations of (A) progesterone (PROG), (B) dehydroepiandrosterone (DHEA), (C) testosterone (T), (D) oestradiol (E_2) and (E) cortisol (CORT) in long-day animals (LD; black), LD animals given timed melatonin injections (LD-M; pink) and animals that were exposed to short-day photoperiods (SD; yellow) following behavioural testing. Data are presented as the mean ± SEM (LD: n = 5 or 6, LD-M: n = 6 or 7, SD: n = 7 or 8). Different lowercase letters indicate a significant difference between treatment groups ($P < 0.05$; PROG and CORT: one-way ANOVAs with Tukey's honestly significant difference post-hoc tests; DHEA, T and E_2: Kruskal-Wallis one-way ANOVAs on ranks with Dunn's post-hoc tests for multiple comparisons). *Outlier excluded from statistical analysis (not shown): one LD-M animal for circulating T concentration*

but there was no significant difference in DHEA concentration in the PAG between LD and LD-M hamsters ($P = 0.153$, $g = -0.494$) (Figure 4C). In addition, there was no effect of treatment on neural PROG levels (PERMANOVA: $F_{1,20} = 1.472$, $P = 0.226$, $R^2 = 0.069$) (Figure 4B).

Conversely, LD-M and SD animals showed reduced levels of biologically active steroids in the brain relative to LD animals (Figure 4D-F). There was a significant effect of treatment on neural T levels (PERMANOVA: $F_{1,20} = 17.47$, $P = 0.002$, $R^2 = 0.466$) (see Supporting information, Table S5), in which LD-M and SD animals had a significantly lower neural T concentration than LD animals in all brain regions of interest (LS [Kruskal-Wallis one-way ANOVA on ranks]: $H = 12.35$, $df = 2$, $P = 0.002$, $\eta^2 = 0.575$; AH [Kruskal-Wallis one-way ANOVA on ranks]: $H = 12.39$, $df = 2$, $P = 0.002$, $\eta^2 = 0.577$; MeA [Kruskal-Wallis one-way ANOVA on ranks]: $H = 8.979$, $df = 2$, $P = 0.011$, $\eta^2 = 0.388$; PAG [Kruskal-Wallis one-way ANOVA on ranks]: $H = 9.211$, $df = 2$, $P = 0.010$, $\eta^2 = 0.401$). Although LD-M and SD animals generally showed similar magnitudes of reduction in neural T, SD animals exhibited a more pronounced reduction in T in the LS than LD-M animals ($P = 0.039$, $g = -0.728$) (Figure 4D). Furthermore, there was a significant effect of treatment on neural E_2 levels (PERMANOVA: $F_{1,20} = 4.445$, $P = 0.036$, $R^2 = 0.181$) (see Supporting information, Table S5). SD animals trended towards a decrease in E_2 concentration in the AH (Kruskal-Wallis one-way ANOVA on ranks: $H = 3.727$, $df = 2$, $P = 0.077$, $g = -0.998$) and PAG (Kruskal-Wallis one-way ANOVA on ranks: $H = 2.363$, $df = 2$, $P = 0.062$, $g = -0.417$) relative to LD animals. There was no significant difference in neural E_2 levels, however, between LD and LD-M animals in either of these regions (AH: $P = 0.401$, $g = -0.431$; PAG: $P = 0.180$, $g = -0.799$). By contrast, there were no significant differences in E_2 concentration in the LS (Kruskal-Wallis one-way ANOVA on ranks: $H = 0.448$, $df = 2$, $P = 0.799$, $\eta^2 = 0.086$) or MeA (Kruskal-Wallis one-way ANOVA on ranks: $H = 0.984$, $df = 2$, $P = 0.612$, $\eta^2 = 0.056$) (Figure 4E) between treatment groups. There was also no effect of treatment on neural CORT levels (one-way MANOVA: $F_{2,12} = 0.709$, $P = 0.681$, partial $\eta^2 = 0.221$) (Figure 4F).

3.6 | Male hamsters showed distinct relationships between aggression and steroid levels across seasonal phenotypes

To assess differences in associations between aggressive behaviour, circulating steroid levels and neurosteroid levels across seasonal phenotypes, correlation matrices consisting of Spearman's ρ values were computed for each treatment group (Figure 5). Based on PERMANOVA, there was a significant effect of treatment on these relationships (PERMANOVA: $F_{1,142} = 11.94$, $P < 0.001$, $R^2 = 0.078$).

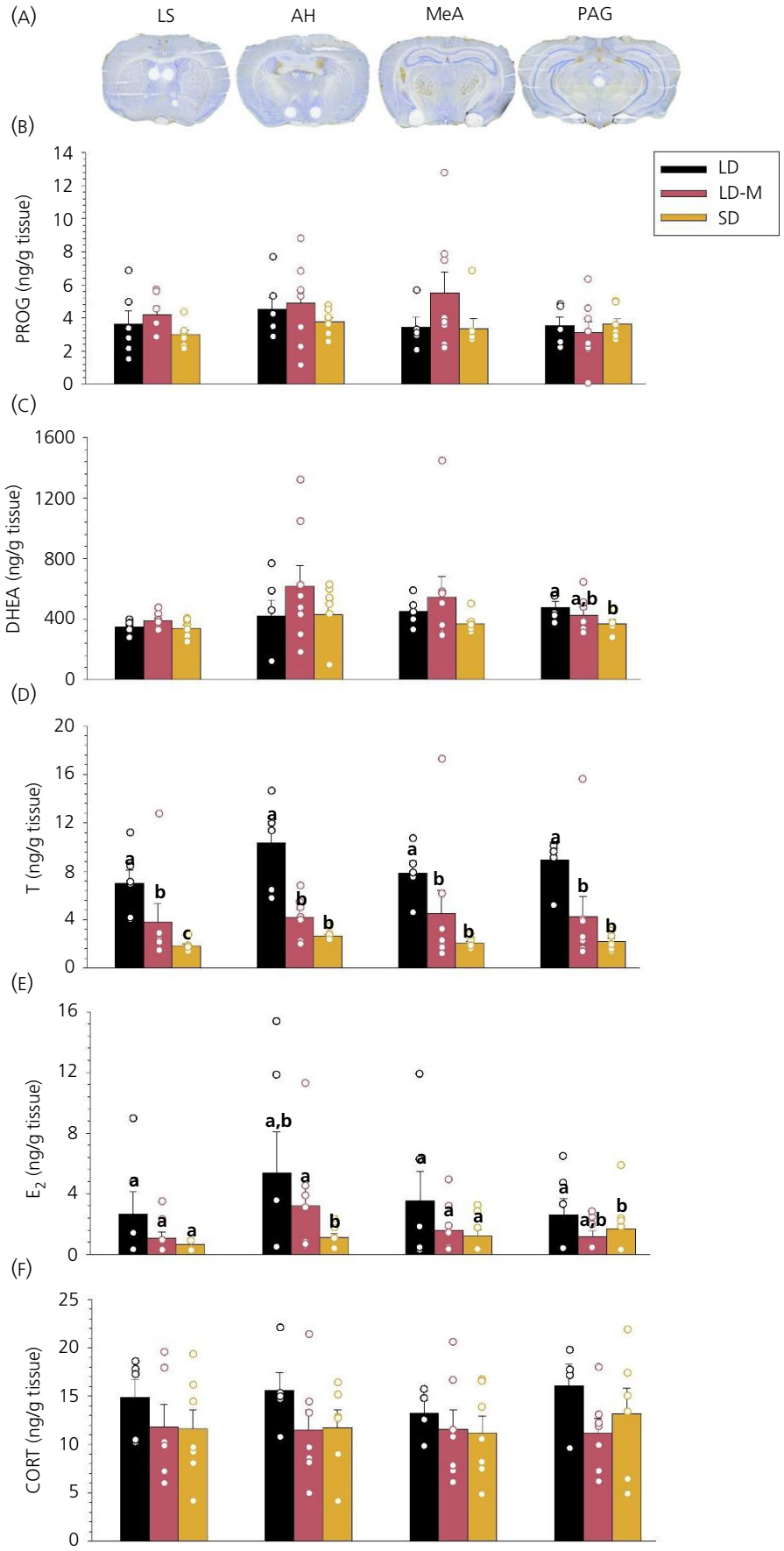

FIGURE 4 Melatonin administration and exposure to short-day photoperiods reduced neural testosterone across all regions of interest, but caused region-specific decreases in dehydroepiandrosterone and oestradiol. (A) Representative coronal sections (100 μm thick, Nissl stained) of Siberian hamster brain illustrating the locations at which micropunches for liquid chromatography-tandem mass spectrometry were acquired. Regions of interest included the lateral septum (LS), anterior hypothalamus (AH), medial amygdala (MeA) and periaqueductal gray (PAG). Concentrations of (B) progesterone (PROG), (C) dehydroepiandrosterone (DHEA), (D) testosterone (T), (E) oestradiol (E_2) and (F) cortisol (CORT) in the LS, AH, MeA and PAG of long-day hamsters (LD; black), LD hamsters given timed melatonin injections (LD-M; pink) and hamsters that were exposed to short-day photoperiods (SD; yellow). Data are presented as the mean ± SEM (LD: n = 4-6, LD-M: n = 6-8, SD: n = 6-8). Different lowercase letters indicate a significant difference between treatment groups ($P < 0.05$; Kruskal-Wallis one-way ANOVAs on ranks with Dunn's post-hoc tests for multiple comparisons). *Outliers excluded from statistical analysis (not shown): 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*

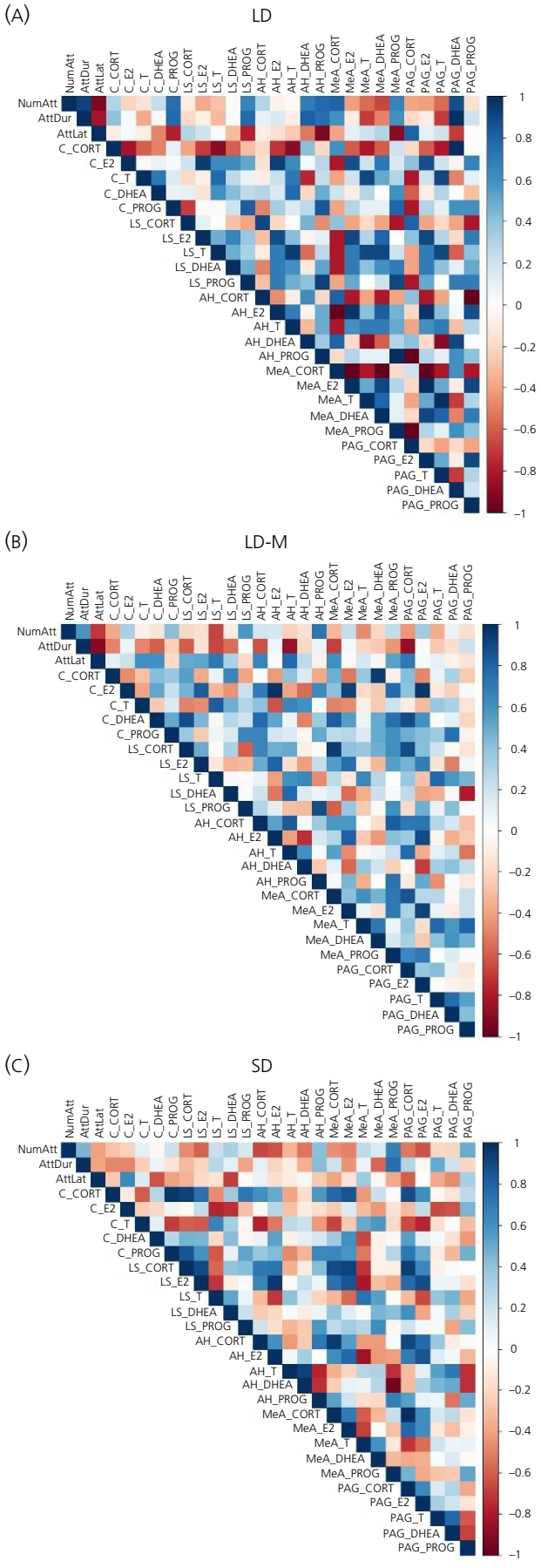

FIGURE 5 Male hamsters treated with melatonin or short-day photoperiods exhibited relationships between aggressive behaviour and steroid levels that differed from those of long day hamsters. Heat maps of Spearman's ρ values for pairwise correlations between aggression and circulating and neural hormone levels in (A) long-day hamsters (LD), (B) LD hamsters given timed melatonin injections (LD-M) and (C) hamsters that were exposed to short-day photoperiods (SD). Correlation coefficients (ρ) are presented on a gradient colour scale, in which positive values are represented in cool colours and negative values are represented in warm colours

Visually and statistically, the correlation matrices of LD-M and SD hamsters were more similar to one another than to LD hamsters.

In general, LD animals exhibited positive associations been levels of prohormones (ie, PROG and DHEA) and aggressive behaviour, although they showed no relationships between levels of biologically active steroids (ie, T, E_2 and CORT) and aggression (Figures 5 and 6; see also Supporting information, Table S6). LD animals displayed positive correlations between number of attacks and PROG levels in the AH (Spearman's rank correlation: $\rho = 0.78$, $n = 6$, $P = 0.07$) (Figure 6C), number of attacks and DHEA levels in the PAG (Spearman's rank correlation: $\rho = 0.87$, $n = 5$, $P = 0.05$), attack duration and PROG levels in the LS and AH (LS [Spearman's rank correlation]: $\rho = 0.77$, $n = 6$, $P = 0.07$; AH [Spearman's rank correlation]: $\rho = 0.75$, $n = 6$, $P = 0.08$) (see Supporting information, Table S6) and attack duration and DHEA levels in the AH and PAG (AH [Spearman's rank correlation]: $\rho = 0.81$, $n = 6$, $P = 0.05$; PAG [Spearman's rank correlation]: $\rho = 1.00$, $n = 5$, $P < 0.01$) (Figure 6E), and displayed negative correlations between attack latency and PROG levels in the LS, AH and MeA (LS [Spearman's rank correlation]: $\rho = -0.77$, $n = 6$, $P = 0.07$; AH [Spearman's rank correlation]: $\rho = -0.93$, $n = 6$, $P = 0.01$; MeA [Spearman's rank correlation]: $\rho = -0.90$, $n = 5$, $P = 0.04$) (see Supporting information, Table S6). LD hamsters also exhibited positive correlations between circulating PROG and aggressive behaviour, in which circulating PROG levels were positively correlated with number of attacks (Spearman's rank correlation: $\rho = 0.75$, $n = 6$, $P = 0.08$) (Figure 6A) and attack duration (Spearman's rank correlation: $\rho = 0.77$, $n = 6$, $P = 0.07$) and were negatively correlated with attack latency (Spearman's rank correlation: $\rho = -0.77$, $n = 6$, $P = 0.07$) (see Supporting information, Table S6).

By contrast, LD-M and SD animals displayed negative correlations between concentrations of biologically active steroids and aggressive behaviour and showed positive correlations between prohormone concentrations and aggression (Figures 5 and 6; see also Supporting information, Table S6). LD-M animals exhibited negative associations between attack duration and T levels in the AH (Spearman's rank correlation: $\rho = -0.86$, $n = 7$, $P = 0.01$) (Figure 6H) and attack duration and CORT levels in the MeA and PAG (MeA [Spearman's rank correlation]: $\rho = -0.68$, $n = 7$, $P = 0.09$; PAG [Spearman's rank correlation]: $\rho = -0.86$, $n = 7$, $P = 0.01$) (see Supporting information, Table S6), but exhibited positive associations between number of attacks and PROG levels in the AH (Spearman's rank correlation: $\rho = 0.73$, $n = 8$, $P = 0.04$) (Figure 6D) and attack

latency and T levels in the AH (Spearman's rank correlation: $\rho = 0.77$, n = 6, $P = 0.07$) (see Supporting information, Table S6). Similarly, SD animals displayed negative correlations between number of attacks and E_2 levels in the AH and PAG (AH [Spearman's rank correlation]: $\rho = -0.64$, n = 8, $P = 0.09$; PAG [Spearman's rank correlation]: $\rho = -0.64$, n = 8, $P = 0.09$) and attack latency and DHEA levels in the LS (Spearman's rank correlation: $\rho = -0.69$, n = 8, $P = 0.06$), but displayed a positive correlation between attack duration and PROG levels in the MeA (Spearman's rank correlation: $\rho = 0.75$, n = 6, $P = 0.08$) (see Supporting information, Table S6). SD animals also exhibited a positive correlation between attack latency and circulating E_2 levels (Spearman's rank correlation: $\rho = 0.64$, n = 8, $P = 0.09$) and a negative correlation between attack latency and circulating DHEA levels (Spearman's rank correlation: $\rho = -0.63$, n = 8, $P = 0.09$) (see Supporting information, Table S6).

4 | DISCUSSION

In the present study, we examined how the hormone melatonin facilitates seasonal changes in neurosteroid levels and aggressive behaviour in male Siberian hamsters using LC-MS/MS. We found that timed melatonin administration induces seasonally appropriate changes in reproductive physiology, peripheral steroidogenesis and aggressive behaviour, including gonadal regression, reductions in EWAT mass and circulating T, and increased aggression. We also showed that LD-M and SD hamsters exhibit region-specific decreases in neural DHEA, T and E_2. Lastly, we demonstrated that male hamsters show distinct relationships between aggression and circulating and neural steroid levels across seasonal phenotypes. Taken together, our findings suggest that melatonin elevates aggressive behaviour, at least in part, by decreasing neurosteroid levels, possibly by modulating the conversion of prohormones to biologically active steroids and steroid receptor signalling within behaviourally relevant brain regions.

4.1 | LC-MS/MS: a sensitive technique for measuring circulating and neural steroid hormones

For the present study, we developed novel steroid extraction and LC-MS/MS protocols that enabled us to measure five steroid hormones (PROG, DHEA, T, E_2 and CORT) in minute amounts of blood (3 μL) and microdissected brain tissue (0.98-4.41 mg) from male Siberian hamsters. Using these methods, we showed that male hamsters have remarkably high levels of DHEA, both in blood and in brain tissue, compared to values reported for other rodent species.[68,71] Unlike traditional rodent models (ie, mice and rats),[72,73] Siberian hamsters express 17β-hydroxylase, which is the enzyme necessary to synthesise adrenal androgens, and secrete significant amounts of DHEA into circulation.[21,22] Because DHEA is capable of crossing the blood-brain-barrier and can be converted to biologically active steroids (eg, T and E_2) in regions that express the appropriate steroidogenic enzymes,[74,75] it is logical that this species has unusually high levels of this steroid in circulation and in the brain. Conversely, we found that E_2 was non-detectable in approximately 52% of blood samples and 58% of brain tissues samples in our study, and concentrations that were above the limit of detection for the LC-MS/MS protocol were often below the limit of quantification. Thus, the E_2 concentrations reported here should be interpreted with caution.

Traditionally, steroid hormone levels have been measured in blood and tissues using antibody-based techniques, such as enzyme immunoassays and radioimmunoassays. Although antibody-based techniques are sensitive, simple and cost-effective, these methods have several disadvantages, including cross-reactivity with closely related steroids other than the hormone of interest and tissue-specific matrix effects.[76] In recent years, mass spectrometry has emerged as a sensitive technique for identifying and quantifying steroid hormones in circulation and in tissues,[77,78] particularly in traditional animal models (ie, mice and rats)[70,71,79,80] and humans.[81,82] In addition to its high specificity for analyte molecules, mass spectrometry is versatile across matrices (eg, blood and tissues) and allows for simultaneous measurement of multiple steroids in a single sample (ie, "steroid profiling").[83,84] To our knowledge, we are among the first researchers to successfully develop a protocol for quantifying steroid hormones in discrete brain nuclei in a non-traditional rodent model, providing high spatial resolution of steroid levels in brain regions that are associated with aggressive behaviour.

4.2 | Melatonin regulates seasonal shifts in aggression and peripheral steroidogenesis

As expected, we showed that male hamsters treated with melatonin exhibited changes in reproductive physiology that are characteristic of SD animals, including gonadal regression and decreases in EWAT mass. LD-M animals, however, showed less pronounced reductions in paired testes mass and EWAT mass than SD animals and, unexpectedly, exhibited no change in body mass over the course of the study. Because prior studies have shown that LD hamsters given long-term timed melatonin injections display a reduction in body mass,[85,86] it is likely that our findings are a result of the duration of photoperiodic treatment chosen for our study, since seasonal changes in energetics and reproductive physiology typically take 10-12 weeks to fully emerge.[25,87] We also found that melatonin administration and SDs increased aggression, but not other non-aggressive social behaviours (eg, investigation, self-grooming), and that exposure to SDs elevated scent marking behaviour. Because Siberian hamsters use scent marking to establish boundaries for their home territories,[88,89] increases in aggressive and scent marking behaviours are consistent with increased territoriality. These findings are in agreement with our previous work in male Siberian hamsters, in which we demonstrated that long-term melatonin administration and exposure to SDs induces gonadal regression, reduces EWAT mass and elevates aggressive behaviour.[23,26,90] Our results are also supported by other studies conducted in seasonally breeding rodents, which showed that exogenous melatonin increases aggression in LD male Syrian

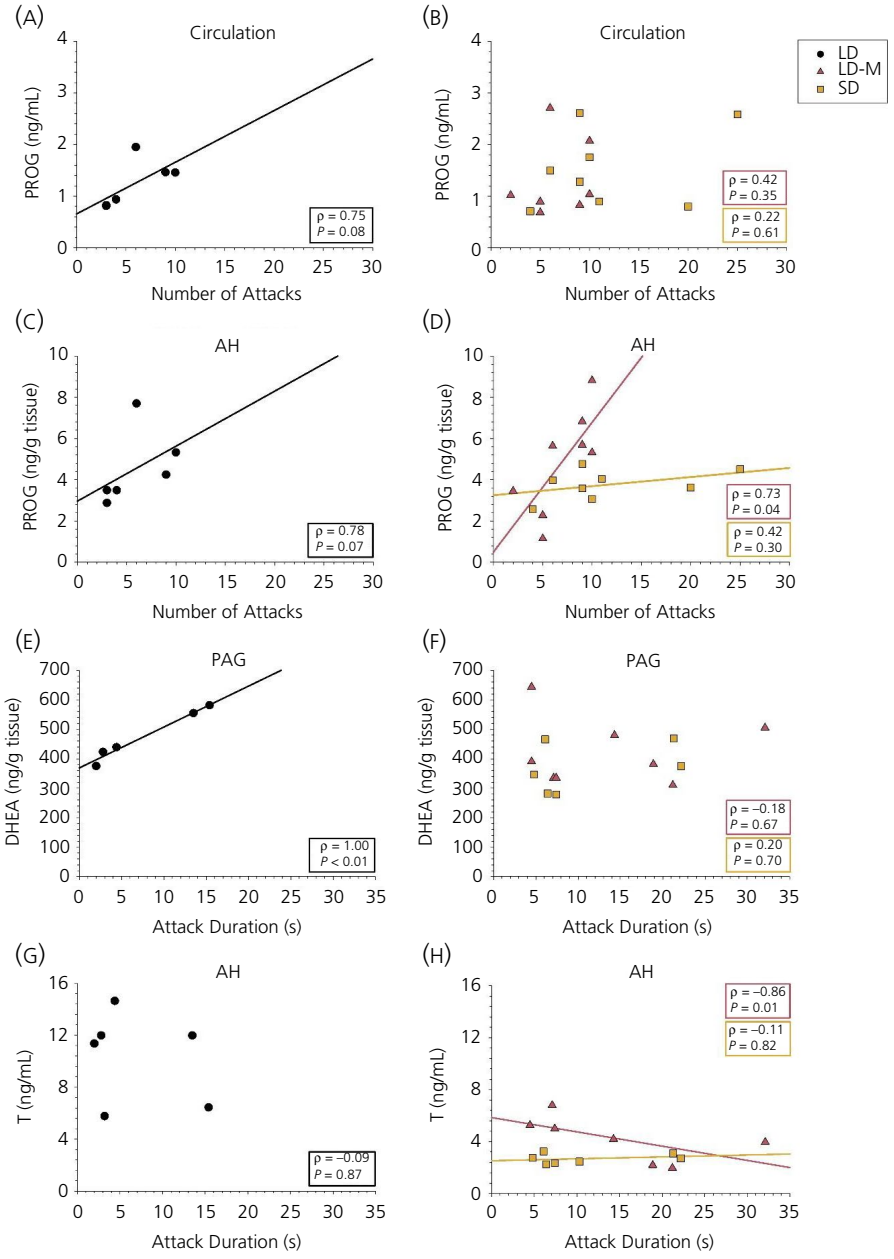

FIGURE 6 Male hamsters administered timed melatonin injections or exposed to short days showed negative relationships between neural levels of biologically active steroids and aggressive behaviour, but exhibited positive relationships between neural prohormone concentrations and aggression. (A) Number of attacks was positively correlated with circulating progesterone (PROG) levels in long-day animals (LD; black circles). (B) Number of attacks and circulating PROG levels were not correlated in LD animals given timed melatonin injections (LD-M; pink triangles) or animals that were exposed to short-day photoperiods (SD; yellow squares). (C) Number of attacks was positively correlated with PROG levels in the anterior hypothalamus (AH) of LD animals. (D) Number of attacks and PROG levels in the AH were positively correlated in LD-M and SD animals. (E) Attack duration and dehydroepiandrosterone (DHEA) levels in the periaqueductal gray (PAG) were positively correlated in LD animals. (F) Attack duration and DHEA levels in the PAG were not correlated in LD-M or SD animals. (G) Attack duration was not correlated with testosterone (T) levels in the AH of LD animals. (H) Attack duration and T levels in the AH were negatively correlated in LD-M and SD animals. Regression lines were generated from Spearman's rank correlations within treatment groups (LD: n = 5 or 6, LD-M: n = 7 or 8, SD: n = 6-8)

hamsters (*Mesocricetus auratus*)[91] and California mice (*Peromyscus californicus*), but that this response was partially blocked in male California mice administered the non-selective melatonin receptor antagonist luzindole.[92] Collectively, these findings suggest that the role of melatonin in regulating aggressive behaviour may be evolutionarily conserved across mammals.

Furthermore, we found that LD-M and SD hamsters displayed reductions in circulating T and SD hamsters trended towards a decrease in circulating DHEA, but showed no change in circulating E_2, CORT or PROG relative to LD hamsters. We also showed that circulating DHEA and E_2 levels were positively correlated with aggressive behaviour in SD hamsters. Importantly, the blood samples analysed in the present study were collected following behavioural testing and, thus, are representative of changes in peripheral steroidogenesis in response to an aggressive encounter. Because SD male hamsters exhibit a reduction in gonadal steroid secretion (ie, T and E_2) and an increase in adrenal DHEA production relative to LD males[26] and, consequently, have higher baseline levels of DHEA and lower baseline levels of T and E_2 in circulation, our results suggest that SD-like patterns of melatonin secretion reduce circulating androgens and elevate circulating oestrogens following an aggressive interaction. These results are consistent with prior studies in Siberian hamsters, in which SD males display decreases in serum DHEA and T levels following an aggressive encounter[26]; SD females exhibit a reduction in serum DHEA and an increase in serum E_2 in response to a social challenge[85,93]; aggression-induced changes in serum DHEA are positively correlated with aggressive behaviour in SD males[26]; and aggression-induced changes in serum DHEA and E_2 are positively correlated with aggression in SD females.[85] Interestingly, while we observed no difference in circulating PROG

levels between treatment groups, we found that circulating PROG was positively correlated with aggression in LD hamsters, but not in LD-M or SD hamsters. Although this is the first study to suggest that circulating PROG is linked to breeding aggression in Siberian hamsters, similar relationships have been shown in other species. Male tree lizards (*Urosaurus ornatus*) and female Syrian hamsters in breeding condition display increased aggression when treated with PROG,[94,95] and female Galápagos marine iguanas (*Amblyrhynchus cristatus*) that display high levels of same-sex aggression during the breeding season have higher levels of circulating PROG.[96] Future studies should characterise the potential mechanisms by which circulating prohormones (ie, PROG and DHEA) modulate seasonal aggression in mammals.

4.3 | Potential mechanisms mediating melatonin-dependent changes in neurosteroid levels

Here, we found that male hamsters given timed melatonin injections or exposed to SDs had lower neural T levels in all regions of interest, including the LS, AH, MeA and PAG, compared to LD hamsters. SD hamsters also had lower DHEA levels in the PAG and lower E_2 levels in the AH and PAG than LD hamsters, yet exhibited no changes in neural PROG or CORT levels. A role for neurosteroids in modulating aggression was further supported by correlation analyses, which showed that LD-M and SD hamsters displayed negative correlations between aggression and biologically active steroids in the AH, MeA and PAG. Interestingly, LD-M and SD hamsters also exhibited positive relationships between neural prohormones (ie, PROG and DHEA) and aggressive behaviour in the LS, AH and MeA, despite the fact that there were few changes in neural PROG or DHEA concentrations in response to treatment. Taken together, these findings suggest that a SD-like melatonin signal induces region-specific reductions in concentrations of androgens and oestrogens in the brain, resulting in increased aggression. These results also support roles for both prohormones and biologically active steroids in elevating aggressive behaviour and suggest that these neuroendocrine circuits are regulated, at least in part, by melatonin.

Because hormonal signalling can be influenced by several mechanisms, including changes in steroid synthesis, metabolism, receptor abundance and binding affinity of steroids for their receptors, it is likely that more than one of these processes may facilitate increased aggression. LD-M and SD hamsters reduced T concentrations in all regions of interest, and SD animals exhibited a reduction in DHEA levels in the PAG, suggesting that these animals may decrease androgen synthesis or increase the conversion of DHEA and/or T to other biologically active steroids, such as E_2 and 5α-dihydrotestosterone (5α-DHT), in these brain regions. Prior studies have reported that non-breeding male song sparrows show increased activity of 3β-hydroxysteroid dehydrogenase and CYP 19 (aromatase), enzymes that catalyse the conversion of DHEA to androstenedione (an active androgen that also serves as a substrate for aromatase) and T to E_2, respectively, but not 5α-reductase, an enzyme that catalyses the conversion of T to 5α-DHT, in brain regions associated with aggression, including the ventromedial telencephalon (containing the nucleus taeniae, a region that is homologous to the mammalian amygdala), ventromedial hypothalamus, central medial telencephalon (containing the lateral septum and bed nucleus of the stria terminalis [BnST]) and/or caudal diencephalon. Unexpectedly, we also found that SD hamsters exhibited reductions in E_2 levels in the AH and PAG. Although these findings are seemingly paradoxical to the observed decreases in neural DHEA and T in these animals, which suggest increased conversion of DHEA to E_2, similar changes have been reported in song sparrows, in which non-breeding males have lower levels of T and E_2 in the preoptic area, AH and nucleus taeniae compared to breeding males.[42] Thus, seasonal decreases in neural E_2 may be the result of reduced synthesis or increased binding of E_2 to its receptors. Indeed, studies have shown that male and female Siberian hamsters, male beach mice and male deer mice exhibit increased oestrogen receptor α expression in brain regions associated with aggression, including the LS, MeA, PAG and/or BnST, during the non-breeding season.[46,47] Interestingly, we showed that LD-M and SD hamsters exhibited significant correlations between aggressive behaviour and CORT and PROG levels in the AH, MeA and PAG, despite showing no changes in the concentrations of these steroids. These results may suggest that, while neural CORT and PROG levels do not vary by season, other components of their signalling pathways, such as changes in receptor abundance or sensitivity, may be important in modulating aggression. Although few studies have investigated the potential roles of progesterone receptor and the two genomic receptors for cortisol, glucocorticoid receptor and mineralocorticoid receptor (GR and MR, respectively), in regulating aggressive behaviour, a population of progesterone receptor-expressing neurones in the ventromedial hypothalamus has been implicated in regulating aggression in male mice.[97,98] In addition, male Syrian hamsters exhibit increased levels of MR, but not GR, in the hypothalamus during SDs.[99] Additional studies are needed to examine whether seasonal changes in the concentrations of other androgens and oestrogens (eg, androstenedione, 5α-DHT, oestriol and oestrone), steroidogenic enzyme activity and steroid receptor signalling modulate aggressive behaviour, as well as how melatonin may mediate these mechanisms.

While our data suggest that melatonin may regulate aggression by altering neurosteroid levels, it is unclear whether the actions of melatonin on aggressive behaviour occur via peripheral and/or central signalling mechanisms. In mammals, circulating melatonin can bind to one of two membrane-bound G protein-coupled receptors: the melatonin 1a receptor (Mel1aR, also known as MT1) and the melatonin 1b receptor (Mel1bR, also known as MT2).[100,101] Of these subtypes, Mel1aR is considered to be primarily responsible for photoperiodic signal transduction[102] because Mel1bR is largely absent from the hypothalamus and pituitary gland of mammals and is absent entirely from Siberian hamsters.[103-105] Mel1aR has been localised in the brain and in peripheral endocrine glands, including the hypothalamus (suprachiasmatic nucleus, paraventricular nucleus, dorsomedial nucleus),[104-106] midbrain (dorsal raphe

nucleus, superior colliculus, substantia nigra),[106,107] hippocampus (dentate gyrus),[106,108] pars tuberalis,[109,110] gonads[111,112] and adrenal glands.[113,114] Because Mel1aR is present in both the brain and periphery, it is possible that melatonin may mediate aggressive behaviour via direct actions on neural substrates (eg, hypothalamus) or via indirect actions on peripheral tissues (eg, adrenal glands and gonads),[21] as observed in other melatonin-dependent mechanisms. For example, lesions of iodomelatonin-responsive cells in the mediobasal hypothalamus block the effects of endogenous and infused melatonin on gonadotrophin secretion in male Syrian hamsters,[115] suggesting that neural melatonin signalling mechanisms mediate seasonal reproduction. Conversely, we have previously shown that melatonin stimulates peripheral steroid synthesis in female hamsters in a seasonal and tissue-specific manner, and that these changes in peripheral steroid levels are associated with increased aggression during the non-breeding season. Specifically, melatonin increases DHEA production in cultured adrenal glands from SD, but not LD females, whereas melatonin elevates DHEA production in cultured ovaries from LD, but not SD females.[27] Collectively, these data suggest that melatonin acts on the adrenal glands to elevate DHEA production and aggression during SDs. Our future work will aim to distinguish how seasonal variation in melatonin signalling, both in the brain and periphery, modulate the neuroendocrine circuits underlying aggressive behaviour.

5 | CONCLUSIONS

In the present study, we demonstrated that male hamsters exhibiting a SD-like pattern of melatonin secretion, either via treatment with timed melatonin injections or exposure to SDs, displayed increased aggression and reduced neurosteroid levels in behaviourally relevant brain regions, a mechanism that likely also involves region-specific changes in steroidogenic enzyme activity, steroid receptor abundance and/or steroid receptor sensitivity. Although the results reported here suggest that melatonin modulates neurosteroid concentrations and aggressive behaviour, additional studies are needed to determine the role of neurosteroids in regulating aggression and to investigate how changes in peripheral and/or central melatonin signalling ultimately lead to increases in aggressive behaviour. Moreover, because several of the brain regions examined in the present study also modulate social behaviours other than aggression (eg, parental care and reproductive behaviours), our future studies will aim to examine whether the neuroendocrine circuits mediating seasonal aggression in this species are distinct from those associated with reproduction. Collectively, the present study enhances our understanding of how melatonin and neurosteroids contribute to aggressive behaviour in mammals.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Kathleen M. Munley: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualisation; Writing – original draft. **Jonathan C. Trinidad:** Formal analysis; Investigation; Methodology; Resources; Validation; Visualisation; Writing-review & editing. **Jessica E. Deyoe:** Conceptualisation; Investigation; Project administration; Supervision; Writing – review & editing. **Catherine H. Adaniya:** Investigation; Writing – review & editing. **Andrea M. Nowakowski:** Investigation; Writing – review & editing. **Clarissa C. Ren:** Investigation; Writing – review & editing. **Grace V. Murphy:** Investigation; Writing – review & editing. **John M. Reinhart:** Investigation; Writing – review & editing. **Gregory E. Demas:** Conceptualisation; Funding acquisition; Project administration; Resources; Supervision; Writing – review & editing.

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DATA AVAILABILITY

Data for this study are available in Mendeley Data (http://dx.doi.org/10.17632/bc7p338xs2.1).

ORCID

Kathleen M. Munley https://orcid.org/0000-0002-7409-3508
Jonathan C. Trinidad https://orcid.org/0000-0002-8279-1509
Clarissa C. Ren https://orcid.org/0000-0001-7719-899X

Gregory E. Demas https://orcid.org/0000-0003-3914-0900

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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