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Sex and seasonal differences in neural steroid sensitivity predict territorial aggression in Siberian hamsters☆

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ABSTRACT

Many animals display marked changes in physiology and behavior on a seasonal timescale, including non-reproductive social behaviors (e.g., aggression). Previous studies from our lab suggest that the pineal hormone melatonin acts via steroid hormones to regulate seasonal aggression in Siberian hamsters (*Phodopus sungorus*), a species in which both males and females display increased non-breeding aggression. The neural actions of melatonin on steroids and aggressive behavior, however, are relatively unexplored. Here, we housed male and female hamsters in long-day photoperiods (LDs, characteristic of breeding season) or short-day photoperiods (SDs, characteristic of non-breeding season) and administered timed melatonin (M) or control injections. Following 10 weeks of treatment, we quantified aggressive behavior and neural steroid sensitivity by measuring the relative mRNA expression of two steroidogenic enzymes (aromatase and 5α-reductase 3) and estrogen receptor 1 in brain regions associated with aggression or reproduction [medial preoptic area (MPOA), anterior hypothalamus (AH), arcuate nucleus (ARC), and periaqueductal gray (PAG)] via quantitative PCR. Although LD-M and SD males and females displayed increased aggression and similar changes in gene expression in the ARC, there were sex-specific effects of treatment with melatonin and SDs on gene expression in the MPOA, AH, and PAG. Furthermore, males and females exhibited different relationships between neural gene expression and aggression in response to melatonin and SDs. Collectively, these findings support a role for melatonin in regulating seasonal variation in neural steroid sensitivity and aggression and reveal how distinct neuroendocrine responses may modulate a similar behavioral phenotype in male and female hamsters.

1. Introduction

Many animals face pronounced fluctuations in environmental conditions on a seasonal basis, including changes in day length (i.e., photoperiod), ambient temperature, and food availability (Bronson and Heideman, 1994; Stevenson et al., 2017). Consequently, most species have evolved a wide range of physiological and behavioral adaptations in response to seasonal variation in their environment, including changes in energy balance, immune function, reproduction, and social behavior, that enable them to prioritize investing in reproduction or survival across the annual cycle (Bartness et al., 2002; Bronson and Heideman, 1994; reviewed in Stearns, 2000; Nelson et al., 2002). To mediate these energetic trade-offs on a seasonal timescale, animals use environmental cues to predict seasonal conditions and adjust their physiology and behavior accordingly. While many factors vary on a seasonal basis, photoperiod is a reliable and relatively 'noise-free' cue from which species can coordinate these seasonal adaptations with the appropriate time of the year (Goldman, 2001; Stevenson et al., 2017; reviewed in Walton et al., 2011). In mammals, the hormone melatonin serves as the biochemical signal from which animals establish and maintain biological rhythms (Goldman, 2001; Stevenson et al., 2017; reviewed in Wood and Loudon, 2014). Photoperiod is converted from an environmental cue into a biochemical signal through a multisynaptic pathway, in which environmental light is perceived by retinal ganglion cells, processed in the hypothalamus, and transduced from a neural to an endocrine signal via the release of melatonin by the pineal gland. Because melatonin production is high at night and low during the day, the pattern of melatonin secretion closely tracks changes in photoperiod

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and, thus, transmits information about day length to the brain and peripheral tissues that are sensitive to melatonin (reviewed in Bartness et al., 1993; Goldman, 2001). Although the role of melatonin in regulating seasonal reproduction is well studied, less is known about how melatonin modulates non-reproductive seasonal social behaviors, such as aggression.

Aggressive behavior is essential in enabling individuals to compete with conspecifics for access to limited resources in their habitat, such as food, territories, and mates (Jalabert et al., 2018; Nelson, 2006). Thus, many species show high levels of aggression during the breeding season, when obtaining a mate and actively defending a territory is critical for enhancing an individual's chances of reproductive success. To date, much of the research conducted on the neuroendocrine processes underlying aggressive behavior has focused extensively on the role of gonadal steroids [e.g., testosterone (T) and estradiol (E_2)] in regulating aggression during the breeding season (reviewed in Cunningham et al., 2012; reviewed in Soma, 2006). Some animals, however, show equivalent or higher levels of aggression during the non-breeding season despite gonadal regression, suggesting that these species face additional selective pressures that favored the evolution of alternative neuroendocrine mechanisms (i.e., mechanisms that are independent of gonadal steroids) to regulate aggressive behavior year-round (reviewed in Munley et al., 2018; reviewed in Soma et al., 2015). Indeed, there is substantial evidence that these species use extra-gonadal sources of steroid hormones, such as the adrenal androgen dehydroepiandrosterone (DHEA) and neurosteroids (i.e., steroids synthesized *de novo* in the brain), to regulate non-breeding aggression. Specifically, seasonal variation in steroid levels, steroidogenic enzymes, and steroid receptors have been demonstrated in the brain and peripheral endocrine tissues of songbirds and rodents that show high levels of aggression during the non-breeding season, including song sparrows (*Melospiza melodia*), spotted antbirds (*Hylophylax n. naevioides*), beach mice (*Peromyscus polionotus*), deer mice (*Peromyscus maniculatus*), and Siberian hamsters (*Phodopus sungorus*; Hau et al., 2004; Jalabert et al., 2021; Munley et al., 2021; Munley et al., 2022c; Pradhan et al., 2010; Rendon et al., 2015; Rendon et al., 2017; Trainor et al., 2007a; Trainor et al., 2007b; Wacker et al., 2010).

Prior research from our group suggests that melatonin modulates seasonal aggression in Siberian hamsters, a solitary species of seasonally breeding rodents in which both males and females exhibit increased aggression during the non-breeding season (Jasnow et al., 2000; Scotti et al., 2007; Wynne-Edwards, 2003). Specifically, our studies suggest that melatonin regulates aggressive behavior by coordinating a 'seasonal switch' from gonadal regulation of aggression during the breeding season to adrenal regulation of aggression during the non-breeding season in male and female Siberian hamsters (reviewed in Munley et al., 2018; Munley et al., 2022b). We have shown that male and female hamsters housed in long-day (LD) photoperiods (characteristic of the breeding season) and given timed melatonin injections, which mimic the pattern of melatonin secretion exhibited by hamsters exposed to short-day (SD) photoperiods (characteristic of the non-breeding season), display higher levels of aggression than LD hamsters given control injections and exhibit SD-like changes in baseline and aggression-induced circulating androgen and estrogen concentrations (Munley et al., 2020; Rendon et al., 2020). Furthermore, treating adrenal glands with melatonin *in vitro* elevates DHEA secretion in SD, but not LD female hamsters, while treating cultured ovaries with melatonin elevates DHEA production in LD, but not SD females (Rendon et al., 2015), suggesting that melatonin acts on the adrenal glands to increase DHEA secretion in non-breeding female hamsters.

Moreover, we have started investigating how melatonin may modulate seasonal aggression via steroid hormones in the brain. We determined that timed melatonin administration and SDs reduce concentrations of DHEA, T, and E_2 in brain regions associated with aggression [lateral septum, anterior hypothalamus (AH), medial amygdala, and/or periaqueductal gray (PAG)] in male hamsters (Munley et al., 2021). We also demonstrated that lentiviral-mediated overexpression of the MT_1 melatonin receptor in the adrenal glands causes SD-like increases in aggression in male hamsters, but does not alter circulating DHEA levels (Munley et al., 2022a), suggesting that adrenal MT_1 receptors control seasonal aggression in male hamsters via neural substrates. In further support of this hypothesis, we recently showed that male and female hamsters exhibit differences in the activity of 3β-hydroxysteroid dehydrogenase, an enzyme that mediates DHEA synthesis and metabolism, in the adrenal glands and AH following treatment with melatonin or SDs (Munley et al., 2022c). Conversely, there is no effect of timed melatonin administration or SDs on the density of neurons expressing aromatase, an enzyme that mediates the conversion of androgens to estrogens, in brain regions associated with aggression or reproduction (the PAG, paraventricular nucleus of the hypothalamus, and ventral tegmental area) in female hamsters (Rendon et al., 2020). While these results suggest that melatonin regulates aggressive behavior via neurosteroids, relatively few studies have examined how steroidogenic enzymes and steroid receptors in the brain may facilitate the actions of melatonin on seasonal aggression and whether these mechanisms may differ between male and female hamsters.

In the present study, we tested the hypothesis that melatonin regulates seasonal aggression by altering neural steroid sensitivity in Siberian hamsters. Specifically, we assessed seasonal and melatonin-dependent changes in neural sensitivity to steroid hormones by quantifying the expression of three steroid-related genes in the brain: 1) cytochrome P450 family 19 subfamily A member 1 (*cyp19a1*), a gene that encodes aromatase, 2) estrogen receptor 1 (*esr1*), a gene that encodes estrogen receptor α, and 3) steroid 5α-reductase type 3 (*srd5a3*), a gene that encodes 5α-reductase 3, an enzyme that mediates the conversion of testosterone to 5α-dihydrotestosterone (DHT). For this study, we chose to measure the expression of genes associated with steroid metabolism and estrogen signaling based on our 'seasonal switch' model for the neuroendocrine regulation of seasonal aggression, which proposes that non-breeding aggression is regulated via the local conversion of DHEA to estrogens (reviewed in Munley et al., 2018; Munley et al., 2022b). We housed male and female hamsters in LDs or SDs, and we administered timed melatonin injections to a subset of LD hamsters, which summated with the endogenous melatonin profile of these hamsters to mimic a SD-like signal. After 10 weeks of treatment, we quantified aggressive and non-aggressive behaviors (e.g., investigation, self-grooming) and measured the relative expression of *cyp19a1*, *esr1*, and *srd5a3* in four brain regions associated with aggression or reproduction: the medial preoptic area (MPOA), a sexually-dimorphic nucleus that has been implicated in modulating sexual behavior and aggression (reviewed in Ball and Balthazart, 2004; Hull and Dominguez, 2012); the arcuate nucleus (ARC) of the hypothalamus, a region that is critical for controlling reproduction and sexual behavior (reviewed in Lehman et al., 2010; Micevych et al., 2015); and the AH and PAG, which are involved in regulating aggressive behavior (reviewed in Lischinsky and Lin, 2020; Nelson and Trainor, 2007; Fig. 1). We hypothesized that melatonin facilitates increased aggression during the non-breeding season by increasing the synthesis of biologically active steroids (i.e., T, E_2, and DHT) and the expression of steroid receptors (i.e., androgen and estrogen receptors) in the brain. Thus, we predicted that male and female hamsters exhibiting a SD-like melatonin signal, either via timed melatonin administration or exposure to SDs, will display higher levels of aggression and will show increases in relative *cyp19a1*, *esr1*, and *srd5a3* expression in brain regions associated with aggression (i.e., MPOA, AH, and PAG), but not in brain regions associated with reproduction (i.e., ARC).

2. Materials and methods

2.1. Experimental animals

Adult male and female Siberian hamsters (*Phodopus sungorus*, >60

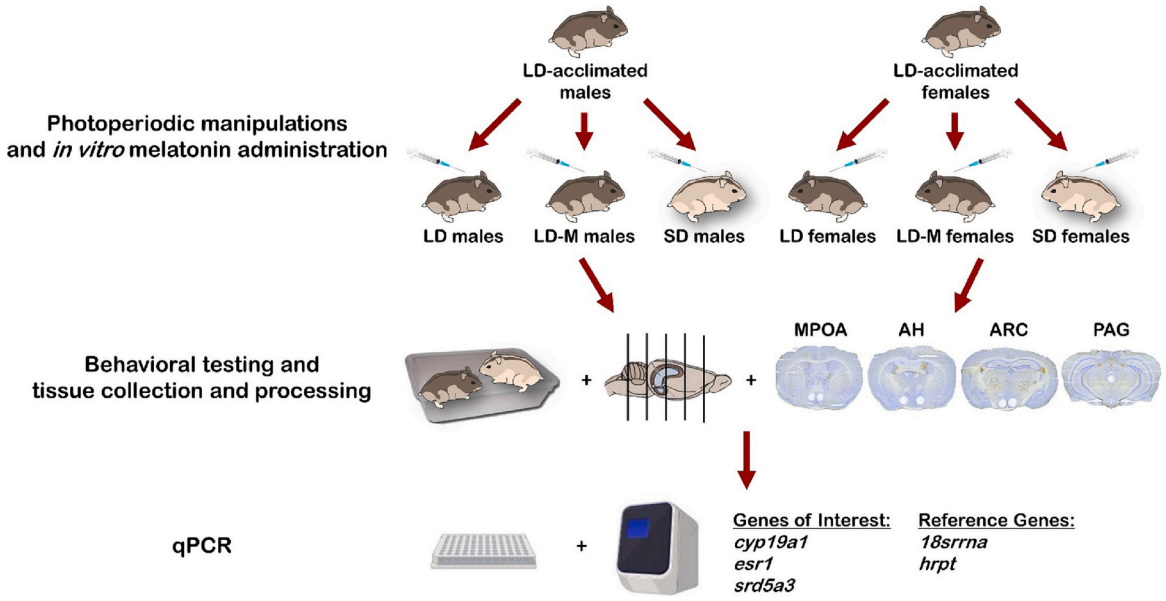

Fig. 1. Schematic of experimental design. Long day (LD)-acclimated male and female hamsters were housed in either LD or short-day (SD) photoperiods. A subset of LD hamsters was administered timed subcutaneous injections of melatonin (M), and all remaining hamsters were given injections of a control solution. Following 10 weeks of photoperiodic treatment and timed melatonin or control injections, aggressive and non-aggressive behaviors were measured using a same-sex resident-intruder paradigm, and brain tissue was collected, sectioned, and microdissected for four brain regions of interest: the medial preoptic area (MPOA), anterior hypothalamus (AH), arcuate nucleus (ARC), and periaqueductal gray (PAG). The relative mRNA expression of cytochrome P450 family 19 subfamily A member 1 (*cyp19a1*), estrogen receptor 1 (*esr1*), steroid 5α-reductase type 3 (*srd5a3*), and the reference genes 18s ribosomal RNA (*18srrna*) and hypoxanthine-guanine phosphoribosyltransferase (*hrpt*) was then quantified in microdissected brain punches via quantitative polymerase chain reaction (qPCR). Figure was created using BioRender.com.

days of age) were raised and maintained in a breeding colony under long-day photoperiods [LDs; light:dark, 16 h:8 h, lights off at 1700 h Eastern Standard Time (EST)] and group-housed at weaning (post-natal day 18) in polypropylene cages (28 × 17 × 12 cm) containing Sani-chip bedding (Teklad, laboratory grade; Envigo, Madison, WI, USA). Hamsters were given *ad libitum* access standard laboratory rodent chow (Teklad global 18 % protein diet; Envigo, Madison, WI, USA) 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

Hamsters (males: $n = 36$, females: $n = 39$) were individually housed for a two-week acclimation period under LDs. Hamsters were then transferred to short-day photoperiods (SDs; males: $n = 20$, females: $n = 20$; 8 L:16 D h, lights on at 0900 h EST) or were kept in LDs (males: $n = 16$, females: $n = 19$). Melatonin profiles were manipulated in a subset of LD hamsters (LD-M; males: $n = 8$, females: $n = 9$), which were administered timed subcutaneous injections of melatonin [15 μg/day (USP Reference Standard; Sigma Aldrich, St. Louis, MO, USA) dissolved in 1:10 ethanol:saline solution]. This protocol was adapted from previously published studies, which demonstrated that a 15 μg/day dose of melatonin injected ≤5 h before lights out induces gonadal regression in male Syrian hamsters when given for 5–12 weeks (Stetson and Tay, 1983) and that this melatonin dose, when given 2 h prior to lights out, reduces body and reproductive tissue mass and increases aggressive behavior in male and female Siberian hamsters when administered for 6–10 weeks (Munley et al., 2020; Munley et al., 2022c; Rendon et al., 2015). All remaining hamsters in the study (males: $n = 28$, females: $n = 30$) were given daily injections of a control (1:10 ethanol:saline) solution. Injections were administered 2 h before lights out (1430–1530 h EST), which extended the LD pattern of endogenous melatonin secretion in LD-M hamsters to mimic that of SD hamsters (Stetson and Tay, 1983). Hamsters remained in photoperiodic treatments and were given injections for 10 weeks.

2.3. Seasonal phenotypes

Following 10 weeks of treatment with timed melatonin or control injections, seasonal phenotypes were determined based on *a priori* criteria that have been previously described for male and female Siberian hamsters (Supplementary Material; Munley et al., 2022a; Rendon et al., 2020). LD hamsters had functional reproductive tissues and showed no significant change in body mass throughout the study. In contrast, LD-M and SD hamsters that were responsive to photoperiodic treatment (males: $n = 12$, females: $n = 12$) had regressed reproductive tissues and showed a significant reduction in body mass (≥5 %). A subset of SD hamsters (males: $n = 8$, 40 %; females: $n = 8$, 40 %) failed to respond to photoperiodic treatment and were classified as "non-responders" (SD-NR) using the same criteria described in the Supplementary Material for LD hamsters. These hamsters were excluded from 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 behaviorally like LD hamsters, has been previously documented in this species and typically affects between 10 and 50 % of the population (Gorman and Zucker, 1997; Puchalski and Lynch, 1986; Rendon et al., 2017).

2.4. Behavioral testing

Social behavior was quantified within the first 3 h of the dark phase (1730–2000 h EST) using a 5 min same-sex resident-intruder paradigm (Supplementary Material; Munley et al., 2020; Rendon et al., 2015). Behavioral interactions were video recorded and scored for aggressive (i.e., latency to first attack, number and duration of attacks and chases) and non-aggressive social behaviors (i.e., frequency and duration of

nose-to-nose investigation, anogenital investigation, and self-grooming) by a single observer (K.M.M.) who was blind to the experimental conditions using Behavioral Observation Research Interactive Software (BORIS) version 7.13.7 (Friard and Gamba, 2016). Measures of aggression, investigation, and grooming were defined according to prior studies on same-sex social behavior in Siberian hamsters (Munley et al., 2020; Scotti et al., 2015). Separate principal component analyses (PCAs) were used: 1) to reduce aggression data (latency to first attack, number and duration of attacks and chases) to a composite 'aggression score' (PC_{Agg}), and 2) to reduce investigation data (frequency and duration of nose-to-nose and anogenital investigation) to a composite 'investigation score' (PC_{Inv}) for each sex. The first principal component of each PCA, which consisted of variables for either aggressive or investigative behaviors, explained a significant proportion of the total variance (males – PC_{Agg}: 61.0 %, PC_{Inv}: 63.0 %; females – PC_{Agg}: 69.0 %, PC_{Inv}: 67.8 %), had a large eigenvalue (>3), and was strongly loaded by most of the variables included in each analysis (i.e., loading values < -0.3 or >0.3; Supplementary Material, Table S1).

2.5. Tissue collection and processing

After behavioral testing (1740–2010 h EST), brains and reproductive tissues were collected from each experimental animal. Animals 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 within 5 min of behavioral testing. Brains were rapidly extracted using RNAse-free tools (< 5 min following euthanasia), collected into sterile polypropylene scintillation vials, flash frozen on dry ice, and stored at −80 °C until processing. Paired testes (for males) and uterine horns and ovaries (for females) were also removed, separated, and weighed individually to the nearest mg.

After collection, brains were covered in Tissue-Tek® O.C.T. compound, and coronal sections (thickness: 250 μm) were cut using a Leica CM 1850 cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on microscope slides. Four brain regions of interest [the medial preoptic area (MPOA), anterior hypothalamus (AH), arcuate nucleus (ARC), and periaqueductal gray (PAG)] were microdissected from both hemispheres with Miltex™ disposable biopsy punches (1 mm diameter, 0.245 mg/punch; Integra LifeSciences, Plainsboro, NJ, USA) using the Palkovits punch technique (Palkovits, 1973). Locations of brain regions were determined using major anatomical landmarks and the mouse brain atlas (Paxinos and Franklin, 2001). Microdissected punches of the MPOA (wet weight: 1.78 ± 0.04 mg), AH (2.11 ± 0.04 mg), ARC (1.35 ± 0.02 mg), and PAG (2.63 ± 0.04 mg) were placed into sterile 2 mL polypropylene microtubes (Sarstedt Inc., Nümbrecht, Germany) containing 5 zirconium ceramic beads (1.4 mm diameter; Fisher Scientific, Waltham, WA, USA) and stored at −80 °C until qPCR analysis. Brain sections were Nissl stained with cresyl violet and examined under a stereo microscope (Leica EZ4; Leica Microsystems, Wetzlar, Germany) to verify the locations of microdissected punches. Representative images of coronal sections depicting the locations of microdissected punches were taken using a Motic EasyScan digital slide scanner (Kowloon, Hong Kong) and are shown in Figs. 1, 3, and 4.

2.6. qPCR

The relative mRNA expression of cytochrome P450 family 19 subfamily A member 1 (*cyp19a1*), estrogen receptor 1 (*esr1*), and steroid 5α-reductase type 3 (*srd5a3*) was measured in microdissected brain tissue via qPCR. Tissue samples were homogenized using a Bead Ruptor 24 Elite (Omni International, Kennesaw, GA, USA), and total RNA was extracted using Maxwell® simplyRNA tissue kits and a Maxwell® Rapid Sample Concentrator instrument (Promega, Madison, WI, USA). RNA quantity was determined using a Take3 micro-volume plate and an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). Complementary DNA (cDNA) was reverse transcribed from 190 ng of total RNA using SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions.

The resulting cDNA was used to measure the mRNA expression of *cyp19a1*, *esr1*, and *srd5a3* and the reference genes 18s ribosomal RNA (*18srrna*) and hypoxanthine-guanine phosphoribosyltransferase (*hrpt*) via qPCR. Custom primer sets were designed for *cyp19a1*, *esr1*, and *srd5a3* using Siberian hamster transcriptome sequences (*cyp19a1* – GenBank accession number: GEVB01004966; *esr1* – GenBank accession number: GEVB01005675; *srd5a3* – GenBank accession number: GEVB01003965; Brekke et al., 2016), and the primer sets for *18srrna* and *hrpt* have been previously published in Siberian hamsters (Banks et al., 2016; Bao et al., 2019; Stewart et al., 2022; Supplementary Material, Table S2). Primer specificity was confirmed for all genes of interest and reference genes via Sanger sequencing, in which successful PCR reactions with Siberian hamster hypothalamic cDNA were run with forward and reverse primers (Quintara Biosciences, San Francisco, CA, USA). All primers were validated using serial dilutions of cDNA from Siberian hamster hypothalamic tissue (replication efficiencies – *cyp19a1*: 99.38 %, *esr1*: 103.57 %, *srd5a3*: 91.96 %, *18srrna*: 102.59 %, *hrpt*: 90.52 %), and melt curves were used to confirm that reactions yielded a single product.

qPCR reactions (10 μL) were run in triplicate alongside no template controls (NTCs) in a QuantStudio™ 6 Flex Real-Time PCR system (Thermo Fisher Scientific, Waltham, WA, USA) using PerfeCTa SYBR Green SuperMix with low ROX (Quanta Biosciences, Gaithersburg, MD, USA). Each well contained 1 μL cDNA diluted 1:10 in nuclease-free water (or 1 μL nuclease-free water for NTCs), 1 μL forward and reverse primers (from 10 μM concentration stocks), 2 μL nuclease-free water, and 5 μL PerfeCTa SYBR Green SuperMix for a total volume of 10 μL. The following thermocycling conditions were used: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 15 s. A final melting stage of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s was run to confirm single-product specificity of each reaction. cDNA samples from different treatment groups were counterbalanced across 12 MicroAmp™ optical 384-well reaction plates (Applied Biosystems, Foster City, CA, USA). Inter-plate and intra-plate variability were determined using a pooled reference sample of Siberian hamster hypothalamic cDNA, which was run on 8 of 12 plates. The inter-plate variability was ≤2.23 % (*cyp19a1*: 0.69 %, *esr1*: 1.25 %, *srd5a3*: 0.91 %, *18srrna*: 0.84 %, *hrpt*: 2.23 %), and the average intra-plate variability was ≤0.86 % (*cyp19a1*: 0.86 ± 0.27 %, *esr1*: 0.50 ± 0.18 %, *srd5a3*: 0.43 ± 0.09 %, *18srrna*: 0.40 ± 0.08 %, *hrpt*: 0.31 ± 0.04 %).

QuantStudio™ Design & Analysis software version 2.6.0 (Thermo Fisher Scientific, Waltham, WA, USA) was used to determine the relative mRNA expression of *cyp19a1*, *esr1*, and *srd5a3* via the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method, in which the mRNA expression of a gene of interest is calculated as the fold-change in expression relative to a calibrator sample and normalized to the expression of one or more reference genes (Schmittgen and Livak, 2008). The geometric mean Ct of *18srrna* and *hrpt* was used as the reference value for calculating ΔCt for a given sample, and a pooled sample of hypothalamic tissue collected from LD male and female Siberian hamsters was used as a calibrator for determining ΔΔCt.

2.7. Statistical analyses

Statistical testing was performed using R version 4.2.1 (R Core Team, 2022), and statistical significance was attributed at $p < 0.05$ after controlling for false discovery rate in the case of multiple comparisons (Verhoeven et al., 2005). A two-way analysis of variance (ANOVA) was used to assess the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on percent change in body mass, and one-way ANOVAs were used to determine the effects of melatonin and photoperiodic treatment on reproductive tissue mass. Permutational multivariate analyses of variance (PERMANOVAs) with 999 permutations were used to evaluate the effects of melatonin and

photoperiodic treatment, sex, and the interaction between treatment and sex on aggression, investigation, and self-grooming based on Euclidean distances. Two-way multivariate analyses of variance (MANOVAs) were used to assess the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on relative *cyp19a1* expression, relative *esr1* expression, and relative *srd5a3* expression across brain regions. If a multivariate test reported a significant effect or a trend towards a significant effect ($p < 0.10$) of treatment, sex, and/or the interaction between treatment and sex for one or more of the variables included in an analysis, generalized linear models (GLMs, for PERMANOVAs only) and post-hoc testing [Tukey's HSD tests for two-way MANOVAs and Dunn's tests for GLMs] were conducted to examine pairwise comparisons (Supplementary Material). Spearman's rank correlations with a Holm-Bonferroni correction for multiple comparisons were computed to examine associations between aggression and neural gene expression by treatment group and sex, and a PERMANOVA with 999 permutations was used to determine whether relationships between aggression and neural gene expression were affected by photoperiodic and melatonin treatment, sex, and the interaction between treatment and sex based on Euclidean distances. For statistical models, R^2 values (for two-way MANOVAs and ANOVAs) and Wald χ^2 values (for GLMs) were calculated to assess goodness of fit, and R^2 (for PERMANOVAs), partial η^2 (for two-way MANOVAs and ANOVAs), Nagelkerke's pseudo-R^2 values (for GLMs), Cohen's d (for Tukey's HSD post-hoc tests), and Hedge's g (for Dunn's post-hoc tests) were determined to estimate effect size (presented in the main text and Supplementary Material; Ellis, 2010; Magee, 1990; Nagelkerke, 1991).

3. Results

3.1. Timed melatonin injections and SDs induced seasonally appropriate changes in energetics and reproduction in male and female hamsters

Timed melatonin administration and exposure to SDs induced characteristic changes in body mass and reproductive physiology in male and female hamsters (Fig. 2A–D). There was a significant effect of treatment, but there was no effect of sex or the interaction between treatment and sex on percent change in body mass (two-way ANOVA: $F_{5,53} = 1.787$, $p = 0.132$, $R^2 = 0.144$; treatment: $p = 0.043$, $\eta^2 = 0.112$; sex: $p = 0.948$, $\eta^2 < 0.001$; interaction: $p = 0.333$, $\eta^2 = 0.041$). SD males showed a significant decrease in percent change in body mass compared to LD males, and LD-M males had a percent change in body mass that was intermediate to that of LD and SD males (Tukey's HSD post-hoc tests – SD vs. LD males: $p = 0.018$, $d = -1.470$, LD-M vs. LD and SD males: $p \leq 0.761$, $d \leq 0.321$; Fig. 2A). LD-M and SD females also displayed a significant reduction in percent change in body mass relative to LD females (Tukey's HSD post-hoc tests: $p \leq 0.049$, $d \leq -0.649$; Fig. 2B). Furthermore, treatment with melatonin and SDs induced gonadal regression. LD-M and SD males exhibited a significant decrease in paired testes mass relative to LD males, and LD-M and SD females showed a significant reduction in reproductive mass compared to LD females (males – one-way ANOVA: $F_{2,25} = 11.16$, $p < 0.001$, $R^2 = 0.472$, $\eta^2 = 0.472$; females – one-way ANOVA: $F_{2,26} = 4.980$, $p = 0.015$, $R^2 = 0.277$, $\eta^2 = 0.277$; Fig. 2C–D).

3.2. Treatment with melatonin and SDs increased aggressive, but not non-aggressive social behaviors in both sexes

Hamsters treated with timed melatonin injections or SDs showed higher levels of aggression than LD hamsters, regardless of sex (Fig. 2E–F; Table 1). There was a significant effect of treatment, but there was no effect of sex or the interaction between treatment and sex on aggressive behavior (PERMANOVA – treatment: $F_{1,57} = 9.656$, $p = 0.002$, $R^2 = 0.192$; sex: $F_{1,57} = 3.244$, $p = 0.120$, $R^2 = 0.054$; interaction: $F_{2,56} = 6.645$, $p = 0.082$, $R^2 = 0.105$; Supplementary Material, Table S3). SD males had a significantly higher PC_{Agg} than LD males, and

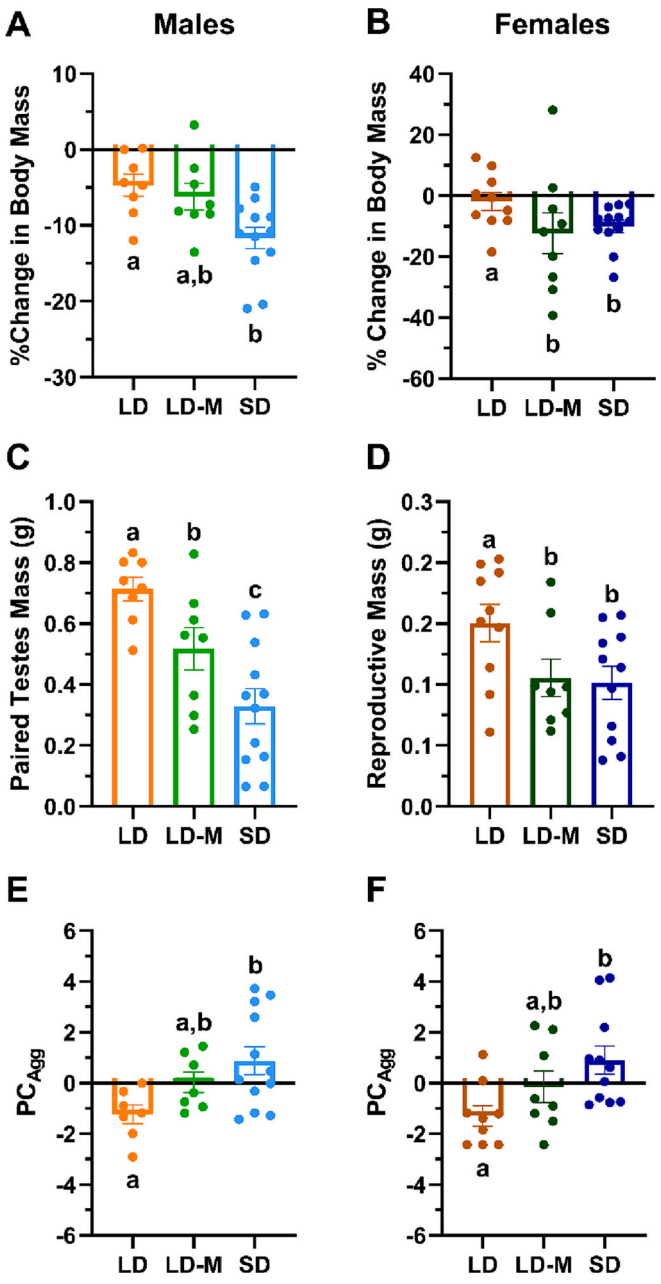

Fig. 2. Treatment with timed melatonin injections and short-day photoperiods reduced body and reproductive tissue mass and increased aggressive behavior in male and female hamsters. (A–B) Percent change in body mass, (C) paired testes mass, (D) reproductive mass, and (E–F) composite aggression score (PC_{Agg}) of long-day hamsters (LD; males: orange, females: rust), LD hamsters given timed melatonin injections (LD-M; males: lime green, females: forest green), and short-day hamsters (SD; males: cerulean, females: indigo). Data are presented as mean ± SEM (males – LD: $n = 8$, LD-M: $n = 8$, SD: $n = 12$; females – LD: $n = 10$; LD-M: $n = 8$–9; SD: $n = 11$–12), and different lowercase 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, reproductive mass, and PC_{Agg}: one-way ANOVAs with Tukey's HSD post-hoc tests).

LD-M males trended towards an increase in PC_{Agg} relative to LD males (one-way ANOVA – $F_{2,25} = 3.453$, $p = 0.047$, $R^2 = 0.217$, $\eta^2 = 0.217$; Tukey's HSD post-hoc tests – SD vs. LD males: $p = 0.018$, $d = 1.113$; LD-M vs. LD males: $p = 0.099$, $d = 0.780$; Fig. 2E). Similarly, SD females showed a significant increase in PC_{Agg} compared to LD females, and LD-M females trended towards an increase in PC_{Agg} relative to LD females

Table 1
Timed melatonin administration and exposure to short-day photoperiods increased territorial aggression in male and female hamsters.

	Males			Females		
	LD	LD-M	SD	LD	LD-M	SD
Number of attacks	12.13 ± 3.543[a]	12.50 ± 2.591[a,b]	22.83 ± 3.863[b]	5.800 ± 1.982[a]	10.00 ± 2.920[a,b]	14.75 ± 3.040[b]
Attack duration	31.71 ± 10.65[a]	32.11 ± 11.17[a]	43.57 ± 9.134[a]	6.768 ± 2.811[a]	11.45 ± 4.971[a,b]	17.17 ± 3.162[b]
Latency to first attack(s)	98.51 ± 32.00[a]	81.53 ± 32.07[a,b]	43.55 ± 7.717[b]	160.0 ± 35.98[a]	61.50 ± 14.82[b]	71.47 ± 15.11[b]
Number of chases	0.375 ± 0.263[a]	2.375 ± 0.822[b]	3.750 ± 1.122[b]	0.600 ± 0.340[a]	2.111 ± 0.841[b]	2.167 ± 0.747[b]
Chase duration (s)	0.157 ± 0.115[a]	1.530 ± 0.543[b]	2.977 ± 1.148[b]	0.378 ± 0.273[a]	1.220 ± 0.513[b]	1.140 ± 0.409[b]

Mean ± SEM (males – LD: $n = 8$, LD-M: $n = 8$, SD: $n = 12$; females – LD: $n = 10$, LD-M: $n = 8$–9, SD: $n = 12$) of number of attacks, attack duration, latency to first attack, number of chases, and chase duration in long-day hamsters (LD), LD hamsters given timed melatonin injections (LD-M), and short-day hamsters (SD) following 10 weeks of treatment. Different lowercase letters (in **bold**) indicate a significant difference between treatment groups within each sex ($p < 0.05$; number of attacks, attack duration, number of chases, and chase duration: generalized linear models with Dunn's post-hoc tests for multiple comparisons, latency to first attack: two-way ANOVA with Tukey's HSD post-hoc tests).

(one-way ANOVA – $F_{2,28} = 2.971$, $p = 0.068$, $R^2 = 0.175$, $\eta^2 = 0.175$; Tukey's HSD post-hoc tests – SD vs. LD females: $p = 0.023$, $d = 1.085$; LD-M vs. LD females: $p = 0.064$, $d = 0.766$; Fig. 2F; Supplementary Material, Table S4). Similar patterns were observed for individual measures of aggressive behavior, in which LD-M and SD hamsters displayed significant increases in number of attacks, number of chases, and chase duration (GLMs, Poisson or negative binomial distributions – $\chi^2(5) > 44.830$, $p \leq 0.261$, $R^2 \leq 0.299$; treatment: $p \leq 0.018$, sex: $p \geq 0.061$, interaction: $p \geq 0.148$) and showed a significant reduction in latency to first attack relative to LD hamsters (two-way ANOVA: $F_{5,52} = 3.140$, $p = 0.015$, $R^2 = 0.232$; treatment: $p = 0.006$, $\eta^2 = 0.174$; sex: $p = 0.203$, $\eta^2 = 0.031$; interaction: $p = 0.281$, $\eta^2 = 0.048$; Table 1; Supplementary Material, Table S4). Conversely, there was no effect of treatment, sex, or the interaction between treatment and sex on investigation or self-grooming (investigation PERMANOVA – treatment: $F_{1,57} = 0.845$, $p = 0.417$, $R^2 = 0.015$; sex: $F_{1,57} = 0.870$, $p = 0.420$, $R^2 = 0.015$; interaction: $F_{2,56} = 0.831$, $p = 0.500$, $R^2 = 0.029$; grooming PERMANOVA – treatment: $F_{1,57} = 0.222$, $p = 0.661$, $R^2 = 0.004$; sex: $F_{1,57} = 2.432$, $p = 0.181$, $R^2 = 0.040$; interaction: $F_{2,56} = 1.717$, $p = 0.170$, $R^2 = 0.058$; Supplementary Material, Table S3).

3.3. Male and female hamsters exhibited regional and sex-specific changes in neural cyp19a1, esr1, and srd5a3 expression in response to timed melatonin administration and SDs

Timed melatonin injections and SDs altered the neural expression of *cyp19a1*, *esr1*, and *srd5a3* in a sex-specific manner (Figs. 3 and 4). There was a significant effect of treatment and sex, but there was no effect of the interaction between treatment and sex on relative *cyp19a1* expression (two-way MANOVA – treatment: $F_{4,34} = 3.745$, $p = 0.013$, $\eta^2 = 0.331$; sex: $F_{4,34} = 4.544$, $p = 0.005$, $\eta^2 = 0.348$; interaction: $F_{4,34} = 1.037$, $p = 0.403$, $\eta^2 = 0.109$; Supplementary Material, Table S5). In the MPOA, there were sex differences in the effects of timed melatonin administration and SDs on relative *cyp19a1* expression. SD males exhibited a significant reduction in relative *cyp19a1* expression in the MPOA relative to LD males, while LD-M males had relative *cyp19a1* expression values in the MPOA that were intermediate to those of LD and SD males (Tukey's HSD post-hoc tests – SD vs. LD males: $p = 0.033$, $d = -1.253$; LD-M vs. LD and SD males: $p \geq 0.139$, $d \geq -0.655$). Conversely, SD females showed a significant increase in *cyp19a1* expression in the MPOA relative to LD females, whereas LD-M females had relative *cyp19a1* expression values in the MPOA that were intermediate to those of LD and SD females (Tukey's HSD post-hoc tests – SD vs. LD females: $p = 0.044$, $d = 0.988$; LD-M vs. LD and SD females: $p \geq 0.143$, $d \leq 0.629$). In the ARC and PAG, LD-M and SD hamsters displayed similar changes in relative *cyp19a1* expression, regardless of sex. SD hamsters showed a significant increase in relative *cyp19a1* expression in the ARC compared to LD hamsters, and LD-M hamsters trended towards an increase in relative *cyp19a1* expression in the ARC relative to LD hamsters (Tukey's HSD post-hoc tests – SD vs. LD hamsters: $p \leq 0.036$, $d \geq 0.997$; LD-M vs. LD hamsters: $p \leq 0.088$, $d \leq 0.782$). In contrast, SD hamsters exhibited a significant decrease in relative *cyp19a1* expression in the PAG compared to LD hamsters, and LD-M hamsters had relative *cyp19a1* expression values in the PAG that were intermediate to those of LD and SD hamsters (Tukey's HSD post-hoc tests – SD vs. LD hamsters: $p \leq 0.041$, $d \leq -0.885$; LD-M vs. LD and SD hamsters: $p \geq 0.111$, $d \leq 0.691$). There was no difference in relative *cyp19a1* expression in the AH, however, between treatment groups in either sex (Tukey's HSD post-hoc tests: $p \geq 0.176$, $d \leq 0.530$; Figs. 3A and 4A; Supplementary Material, Table S6).

Moreover, there was a trend towards an effect of sex, but there was no effect of treatment or the interaction between treatment and sex on relative *esr1* expression (two-way MANOVA – treatment $F_{4,41} = 0.961$, $p = 0.439$, $\eta^2 = 0.087$; sex: $F_{4,41} = 2.552$, $p = 0.053$, $\eta^2 = 0.199$; interaction: $F_{4,41} = 1.288$, $p = 0.291$, $\eta^2 = 0.112$; Supplementary Material, Table S5). SD females displayed a significant increase in relative *esr1* expression in the MPOA relative to LD females, whereas LD-M females had relative *esr1* expression values in the MPOA that were intermediate to those of LD and SD females (Tukey's HSD post-hoc tests – SD vs. LD females: $p = 0.047$, $d = 1.005$; LD-M vs. LD and SD females: $p \geq 0.252$, $d \leq 0.640$). There was no significant difference in relative *esr1* expression in the MPOA, however, between treatment groups in males (Tukey's HSD post-hoc tests: $p \geq 0.659$, $d \leq 0.174$). In the ARC, male and female hamsters showed similar changes in relative *esr1* expression in response to treatment with melatonin and SDs. SD males showed a significant reduction in relative *esr1* expression in the ARC compared to LD males, and LD-M males had relative *esr1* expression values in the ARC that were intermediate to those of LD and SD males (Tukey's HSD post-hoc tests – SD vs. LD males: $p = 0.029$, $d = -1.788$; LD-M vs. LD and SD males: $p \geq 0.267$, $d \geq -0.726$). Similarly, LD-M and SD females exhibited a significant decrease in relative *esr1* expression in the ARC compared to LD females (Tukey's HSD post-hoc tests: $p \leq 0.044$, $d \leq -0.405$). There was no significant difference in relative *esr1* expression in the AH or PAG, however, between treatment groups in either sex (Tukey's HSD post-hoc tests: $p \geq 0.329$, $d \leq 0.536$; Figs. 3B and 4B; Supplementary Material, Table S6).

Finally, there was a significant effect of the interaction between treatment and sex, but there was no effect of treatment or sex on relative *srd5a3* expression (two-way MANOVA – treatment: $F_{4,45} = 0.702$, $p = 0.595$, $\eta^2 = 0.059$; sex: $F_{4,45} = 0.192$, $p = 0.941$, $\eta^2 = 0.017$; interaction: $F_{4,45} = 2.601$, $p = 0.048$, $\eta^2 = 0.188$; Supplementary Material, Table S5). There were sex-specific effects of timed melatonin administration and exposure to SDs on relative *srd5a3* expression in the AH and PAG. SD males displayed a significant increase in relative *srd5a3* expression in the AH compared to LD males, whereas LD-M males had relative *srd5a3* expression values in the AH that were intermediate to those of LD and SD males (Tukey's HSD post-hoc tests – SD vs. LD males: $p = 0.016$, $d = 2.474$; LD-M vs. LD and SD males: $p \geq 0.636$, $d \leq 1.021$). Conversely, there was no effect of treatment on relative *srd5a3* expression in the AH in females (Tukey's HSD post-hoc tests: $p \geq 0.251$, $d \leq 0.970$). In the PAG, LD-M and SD females showed a significant decrease in relative *srd5a3* expression relative to LD females (Tukey's HSD post-hoc tests: $p \leq 0.011$, $d \geq 1.219$). There was no effect of treatment, however, on relative *srd5a3* expression in the PAG in males (Tukey's HSD post-hoc tests: $p \geq 0.312$, $d \leq 0.708$). In addition, there was no significant

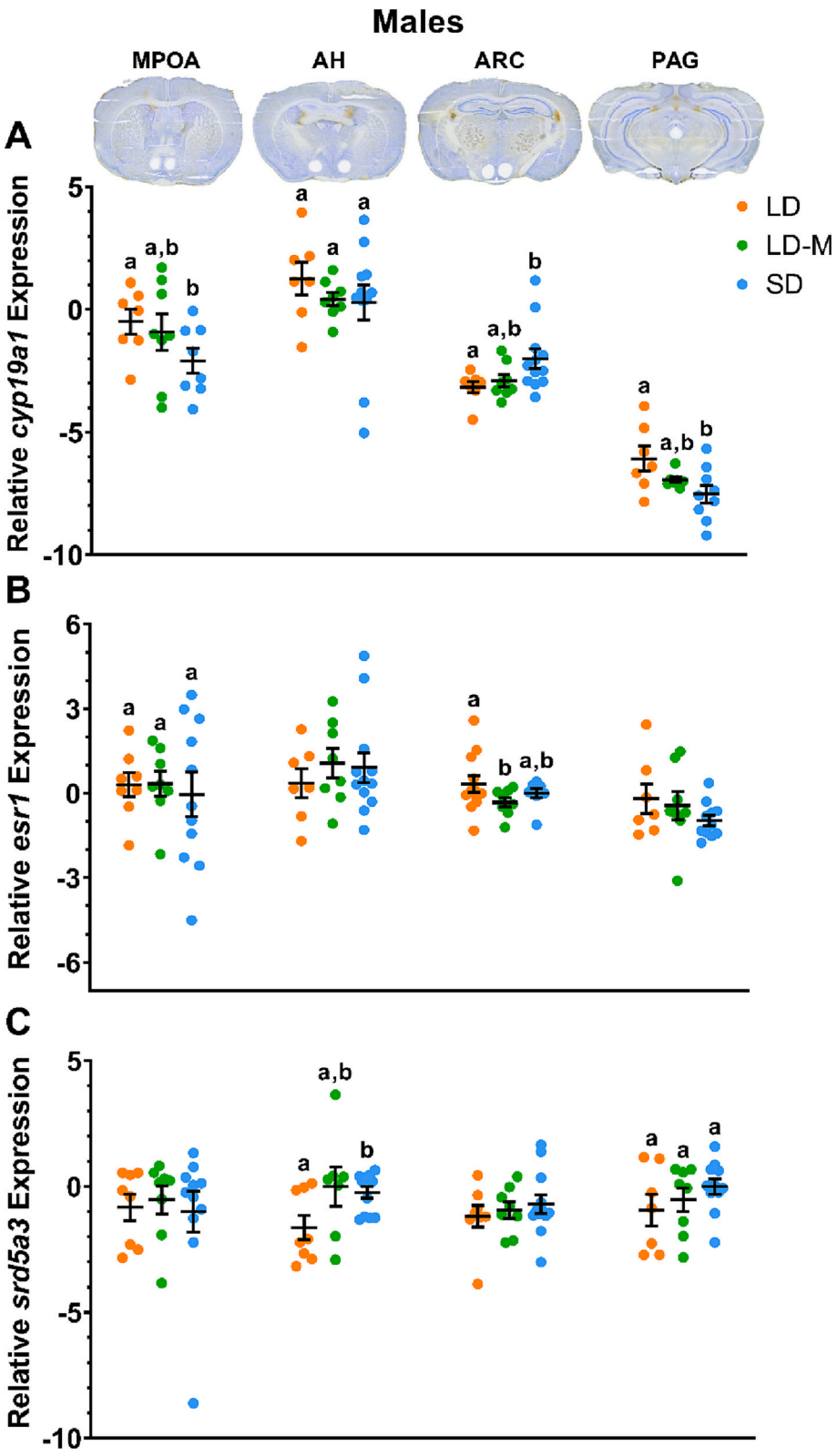

Fig. 3. Male hamsters given timed melatonin injections and exposed to short-day photoperiods show region-specific changes in the neural expression of steroid-related genes. (A) Relative aromatase (*cyp19a1*) expression, (B) relative estrogen receptor 1 (*esr1*) expression, and (C) relative steroid 5α-reductase type 3 (*srd5a3*) expression in the medial preoptic area (MPOA), anterior hypothalamus (AH), arcuate nucleus (ARC), and periaqueductal gray (PAG) of long-day male hamsters (LD; orange), LD males given timed melatonin injections (LD-M; lime green), and short-day males (SD; cerulean). Data are presented as mean ± SEM (LD: n = 7–8, LD-M: n = 7–8, SD: n = 8–12), and different lowercase letters indicate a significant difference between treatment groups within each brain region ($p < 0.05$; two-way ANOVAs with Tukey's HSD post-hoc tests).

difference in relative *srd5a3* expression in the MPOA and ARC between treatment groups in either sex (Tukey's HSD post-hoc tests: $p \geq 0.251$, $d \leq 0.376$; Figs. 3C and 4C; Supplementary Material, Table S6).

3.4. Male and female hamsters displayed seasonal and sex differences in associations between aggression and neural gene expression

To characterize the effects of treatment with melatonin and SDs on relationships between aggression and the neural expression of *cyp19a1*, *esr1*, and *srd5a3* in male and female hamsters, Spearman's rank

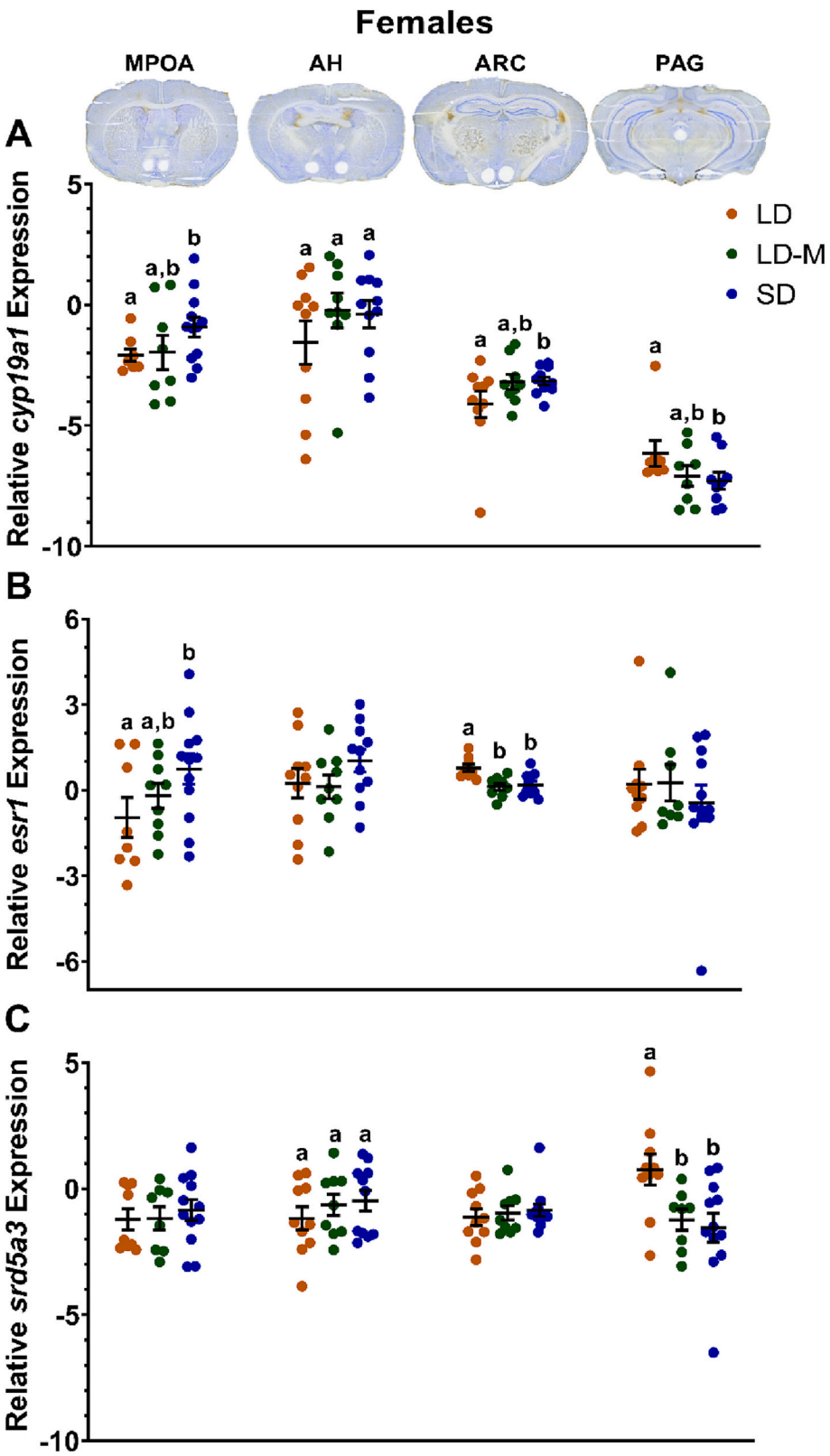

Fig. 4. Female hamsters treated with melatonin and short days display regional variation in the expression of steroidogenic enzymes and estrogen receptor 1 in the brain. (A) Relative aromatase (*cyp19a1*) expression, (B) relative estrogen receptor 1 (*esr1*) expression, and (C) relative steroid 5α-reductase type 3 (*srd5a3*) expression in the medial preoptic area (MPOA), anterior hypothalamus (AH), arcuate nucleus (ARC), and periaqueductal gray (PAG) of long-day female hamsters (LD; rust), LD females given timed melatonin injections (LD-M; forest green), and short-day females (SD; indigo). Data are presented as mean ± SEM (LD: $n = 8$–10, LD-M: $n = 8$–9, SD: $n = 9$–12), and different lowercase letters indicate a significant difference between treatment groups within each brain region ($p < 0.05$; two-way ANOVAs with Tukey's HSD post-hoc tests).

correlations were computed for each treatment group and sex (Fig. 5). There was a significant effect of treatment and sex and there was a trend towards an effect of the interaction between treatment and sex on Spearman's ρ values computed from this analysis (PERMANOVA – treatment: $F_{1,104} = 20.326$, $p < 0.001$, $R^2 = 0.151$; sex: $F_{1,104} = 8.595$, $p < 0.001$, $R^2 = 0.064$; interaction: $F_{2,103} = 1.870$, $p = 0.070$, $R^2 = 0.014$). These results suggest that there are sex-specific effects of timed melatonin administration and exposure to SDs on associations between aggressive behavior and the neural expression of steroid-related genes in Siberian hamsters.

In male and female hamsters, correlations between aggression and neural gene expression varied by treatment and brain region (Figs. 5 and

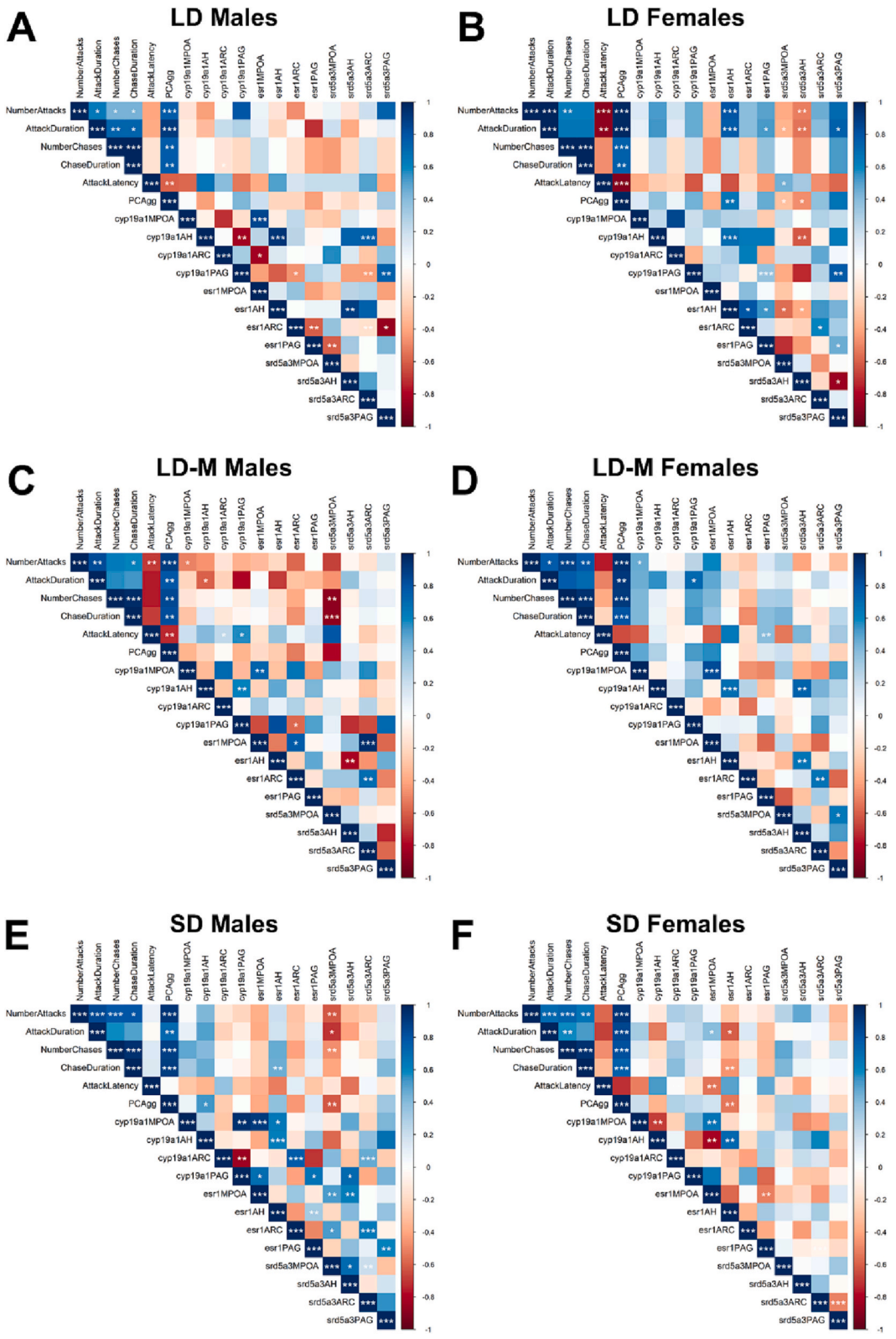

Fig. 5. Hamsters administered timed melatonin injections and exposed to short-day photoperiods exhibit treatment- and sex-specific associations between aggressive behavior and the expression of steroid-related genes in the brain. Heat maps of Spearman's ρ values for pairwise correlations between aggression and the neural expression of steroidogenic enzymes and estrogen receptor 1 in (A–B) long-day hamsters (LD), (C–D) LD hamsters given timed melatonin injections (LD-M), and (E–F) short-day hamsters (SD). Correlation coefficients (ρ) are presented on a gradient color scale, in which positive values are represented in cool colors and negative values are represented in warm colors, and asterisks indicate correlations that are significant ($p < 0.05$) or trending towards significance ($p < 0.10$). Abbreviations: AH, anterior hypothalamus; ARC, arcuate nucleus; cyp19a1, aromatase; esr1, estrogen receptor 1; MPOA, medial preoptic area; PAG, periaqueductal gray; PC_{Agg}, composite aggression score; srd5a3, steroid 5α-reductase type 3. Symbols: $*p < 0.10$, $**p < 0.05$, $***p < 0.01$.

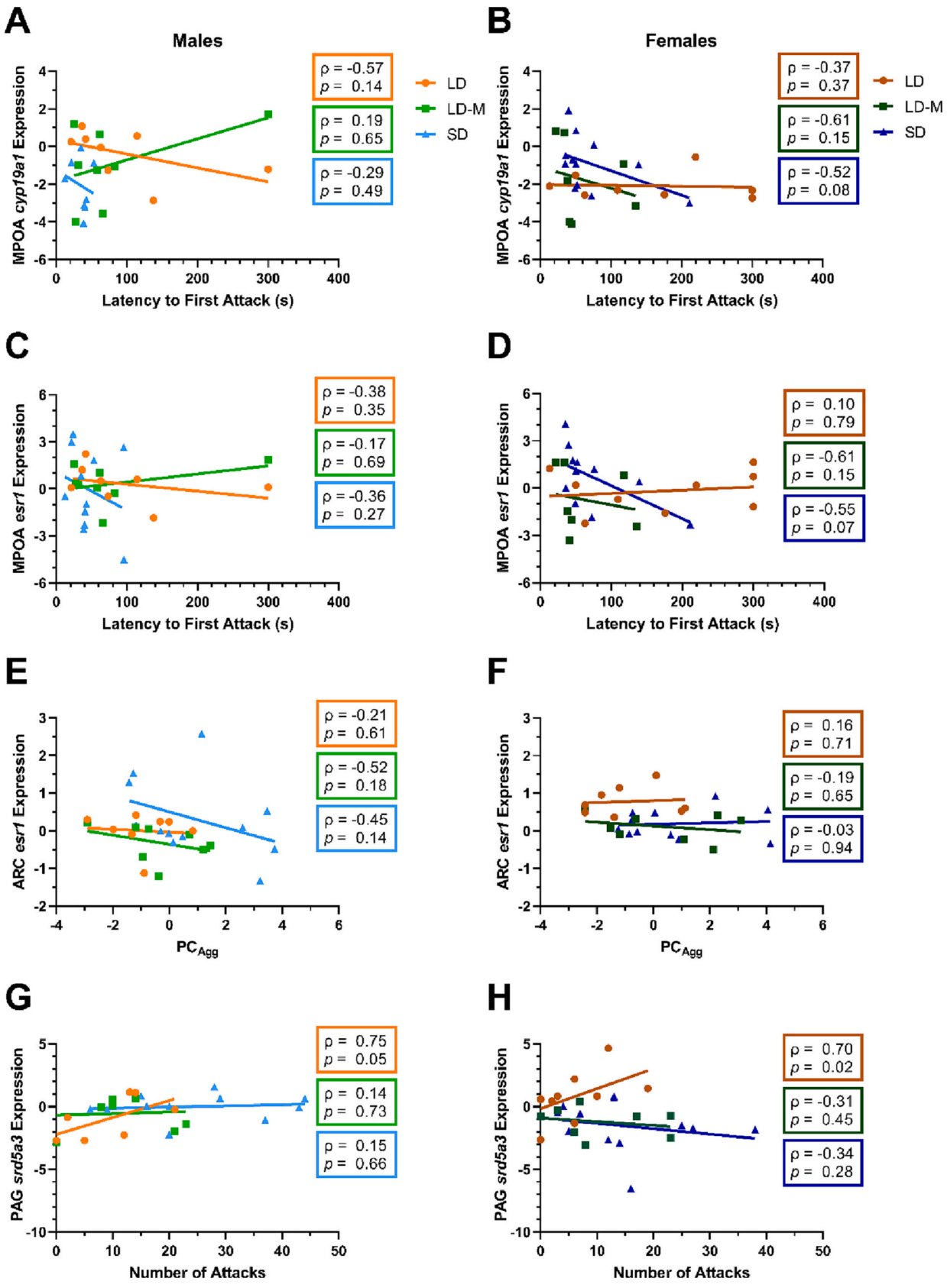

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Fig. 6. Relationships between aggression and the neural expression of steroid-related genes in hamsters vary by sex and in response to treatment with melatonin and short days. (A) Relative aromatase (*cyp19a1*) expression in the medial preoptic area (MPOA) was negatively correlated with latency to first attack in long-day males (LD; orange circles), but not in long-day males given timed melatonin injections (LD-M; lime green squares) or short-day males (SD; cerulean triangles) (B) Relative *cyp19a1* expression in the MPOA was negatively correlated with latency to first attack in LD females (rust circles), LD-M females (forest green squares), and SD females (indigo triangles). (C) Relative estrogen receptor 1 (*esr1*) expression in the MPOA was negatively associated with latency to first attack in LD and SD males, but not in LD-M males. (D) Relative *esr1* expression in the MPOA was negatively associated with latency to first attack in LD-M and SD females, but not in LD females. (E) Relative *esr1* expression in the arcuate nucleus (ARC) was negatively correlated with composite aggression score (PC_{Agg}) in LD-M and SD males, but not in LD males. (F) Relative *esr1* expression in the ARC was not correlated with PC_{Agg} in females, regardless of treatment. (G) Relative steroid 5α-reductase type 3 (*srd5a3*) expression in the periaqueductal gray (PAG) was positively associated with number of attacks in LD males, but not in LD-M or SD males. (H) Relative *srd5a3* expression in the PAG was positively associated with number of attacks in LD females, but was negatively associated with number of attacks in LD-M and SD females. Regression lines were generated from Spearman's rank correlations within treatment groups (males – LD: $n = 7$–8, LD-M: $n = 8$, SD: $n = 8$–12; females – LD: $n = 8$–10; LD-M: $n = 7$–8; SD: $n = 11$–12).

6; Supplementary Material, Table S7). Although males showed few correlations between aggression and relative *cyp19a1* expression in the MPOA, SD males displayed negative correlations between aggressive behavior and relative *esr1* expression in the MPOA, and LD-M and SD males displayed negative correlations between aggression and relative *srd5a3* expression in the MPOA (Spearman's rank correlations – aggression and *cyp19a1* expression, all males: $\rho \leq |0.57|$, $n = 7$–12, $p \geq 0.14$; number of attacks and *esr1* expression, SD males: $\rho = -0.38$, $n = 11$, $p = 0.25$; PC_{Agg} and *esr1* expression, SD males: $\rho = -0.39$, $n = 11$, $p = 0.23$; number of attacks and *srd5a3* expression, LD-M and SD males: $\rho \leq -0.62$, $n = 8$–11, $p \leq 0.07$; PC_{Agg} and *srd5a3* expression, LD-M and SD males: $\rho \leq -0.62$, $n = 8$–11, $p \leq 0.04$; Fig. 6A, C). In contrast, females displayed positive correlations between aggression and relative *cyp19a1* expression in the MPOA, regardless of treatment (Spearman's rank correlations – latency to first attack: $\rho \leq -0.37$, $n = 8$–12, $p \leq 0.37$; Fig. 6B). LD females showed negative associations between aggression and relative *esr1* and *srd5a3* expression in the MPOA (Spearman's rank correlations – chase duration and *esr1* expression: $\rho = -0.46$, $n = 9$, $p = 0.22$; PC_{Agg} and *esr1* expression: $\rho = -0.37$, $n = 9$, $p = 0.32$; number of attacks and *srd5a3* expression: $\rho = -0.39$, $n = 9$, $p = 0.30$; latency to first attack and *srd5a3* expression: $\rho = 0.44$, $n = 9$, $p = 0.24$). Conversely, LD-M and SD females showed positive associations between aggression and relative *esr1* and *srd5a3* expression in the MPOA (Spearman's rank correlations – attack duration and *esr1* expression: $\rho \geq 0.40$, $n = 8$–12, $p \leq 0.23$; latency to first attack and *esr1* expression: $\rho \leq -0.55$, $n = 8$–12, $p \leq 0.15$; chase duration and *srd5a3* expression: $\rho \geq 0.43$, $n = 8$–12, $p \leq 0.28$; Fig. 6D; Supplementary Material, Table S7).

In the AH, SD males exhibited positive relationships between aggression and relative *cyp19a1* and *esr1* expression (Spearman's rank correlations – attack duration and *cyp19a1* expression: $\rho = 0.50$, $n = 12$, $p = 0.10$; PC_{Agg} and *cyp19a1* expression: $\rho = 0.54$, $n = 12$, $p = 0.07$; latency to first attack and *esr1* expression: $\rho = -0.52$, $n = 12$, $p = 0.08$; number of chases and *esr1* expression: $\rho = 0.47$, $n = 12$, $p = 0.13$), while LD males exhibited negative relationships between aggression and relative *cyp19a1* and *esr1* expression (Spearman's rank correlations – latency to first attack and *cyp19a1* expression: $\rho = 0.68$, $n = 7$, $p = 0.09$; PC_{Agg} and *cyp19a1* expression: $\rho = -0.43$, $n = 7$, $p = 0.34$; latency to first attack and *esr1* expression: $\rho = 0.50$, $n = 7$, $p = 0.25$). LD females displayed positive associations between aggression and relative *cyp19a1* and *esr1* expression in the AH (Spearman's rank correlations – number of attacks and *cyp19a1* expression: $\rho = 0.51$, $n = 10$, $p = 0.13$; attack duration and *cyp19a1* expression: $\rho = 0.53$, $n = 10$, $p = 0.11$; number of attacks and *esr1* expression: $\rho = 0.79$, $n = 10$, $p = 0.01$; attack duration and *esr1* expression: $\rho = 0.77$, $n = 10$, $p = 0.01$). In contrast, SD females displayed negative associations between aggression and relative *cyp19a1* expression in the AH, and LD-M and SD females displayed negative associations between aggression and relative *esr1* expression in the AH (Spearman's rank correlations – attack duration and *cyp19a1* expression, SD females: $\rho = -0.53$, $n = 11$, $p = 0.10$; latency to first attack and *cyp19a1* expression, SD females: $\rho = 0.53$, $n = 11$, $p = 0.09$; attack duration and *esr1* expression, SD females: $\rho = -0.61$, $n = 11$, $p = 0.05$; latency to first attack and *esr1* expression, LD-M females: $\rho = 0.64$, $n = 9$, $p = 0.09$). Furthermore, LD females exhibited negative correlations between aggression and relative *srd5a3* expression in the AH (Spearman's rank correlations – number of attacks: $\rho = -0.51$, $n = 10$, $p = 0.13$; PC_{Agg}: $\rho = -0.47$, $n = 10$, $p = 0.17$), while SD females exhibited positive correlations between aggression and relative *srd5a3* expression in the AH (Spearman's rank correlations – number of attacks: $\rho = 0.48$, $n = 11$, $p = 0.14$; latency to first attack: $\rho = -0.38$, $n = 11$, $p = 0.25$; Supplementary Material, Table S7).

In the ARC, male hamsters exhibited negative relationships between aggression and relative *esr1* expression, regardless of treatment (Spearman's rank correlations – number of attacks: $\rho \leq -0.30$, $n = 8$–12, $p \leq 0.35$; Fig. 6E). There were no relationships, however, between aggression and relative *cyp19a1* and *srd5a3* expression in the ARC in male hamsters (Spearman's rank correlations – $\rho \leq |0.39|$, $n = 7$–12, $p \geq 0.38$). Conversely, LD females showed positive relationships between aggression and relative *srd5a3* expression in the ARC (Spearman's rank correlations – attack duration: $\rho = 0.40$, $n = 10$, $p = 0.25$; latency to first attack: $\rho = -0.47$, $n = 10$, $p = 0.17$), whereas LD-M and SD females showed no relationships between aggression and relative *srd5a3* expression in the ARC (Spearman's rank correlations – $\rho \leq |0.23|$, $n = 9$–12, $p \geq 0.55$). There were no associations between aggression and relative *cyp19a1* and *esr1* expression in the ARC in female hamsters (Spearman's rank correlations – $\rho \leq |0.40|$, $n = 8$–11, $p \geq 0.33$; Fig. 6F; Supplementary Material, Table S7).

In the PAG, LD males exhibited negative correlations between aggression and relative *esr1* expression, but displayed positive correlations between aggression and relative *cyp19a1* and *srd5a3* expression (Spearman's rank correlations – attack duration and *esr1* expression: $\rho = -0.71$, $n = 7$, $p = 0.07$; PC_{Agg} and *esr1* expression: $\rho = -0.43$, $n = 7$, $p = 0.34$; number of attacks and *cyp19a1* expression: $\rho = 0.79$, $n = 7$, $p = 0.04$; latency to first attack and *cyp19a1* expression: $\rho = -0.54$, $n = 7$, $p = 0.22$; latency to first attack and *srd5a3* expression: $\rho = -0.39$, $n = 7$, $p = 0.38$; PC_{Agg} and *srd5a3* expression: $\rho = 0.75$, $n = 7$, $p = 0.05$; Fig. 6G). Conversely, LD-M males showed negative correlations between aggression and relative *cyp19a1* expression in the PAG, whereas LD-M and SD males showed no correlations between aggression and relative *esr1* and *srd5a3* expression in the PAG (Spearman's rank correlations – attack duration and *cyp19a1* expression, LD-M males: $\rho = -0.79$, $n = 7$, $p = 0.04$; latency to first attack and *cyp19a1* expression, LD-M males: $\rho = 0.57$, $n = 7$, $p = 0.18$; aggression and *esr1* and *srd5a3* expression, LD-M and SD males: $\rho \leq |0.34|$, $n = 8$–11, $p \geq 0.31$). LD females displayed positive correlations between aggression and relative *cyp19a1*, *esr1*, and *srd5a3* expression in the PAG (Spearman's rank correlations – number of attacks and *cyp19a1* expression: $\rho = 0.47$, $n = 8$, $p = 0.24$; latency to first attack and *cyp19a1* expression: $\rho = -0.61$, $n = 8$, $p = 0.11$; attack duration and *esr1* expression: $\rho = 0.52$, $n = 10$, $p = 0.12$; PC_{Agg} and *esr1* expression: $\rho = 0.46$, $n = 10$, $p = 0.18$; latency to first attack and *srd5a3* expression: $\rho = -0.57$, $n = 10$, $p = 0.08$; PC_{Agg} and *srd5a3* expression: $\rho = 0.67$, $n = 10$, $p = 0.03$). In contrast, SD females displayed negative correlations between aggression and relative *esr1* expression in the PAG, whereas LD-M and SD females displayed no correlations between aggression and relative *srd5a3* expression in the PAG (Spearman's rank correlations – latency to first attack and *esr1* expression, SD females: $\rho = 0.48$, $n = 12$, $p = 0.12$; number of chases and *esr1* expression, SD

females: $\rho = -0.33$, $n = 12$, $p = 0.29$; aggression and *srd5a3* expression, LD-M and SD females: $\rho \leq |0.34|$, $n = 8–12$, $p \geq 0.28$; Fig. 6H; Supplementary Material, Table S7).

4. Discussion

Prior studies from our group suggest that the pineal hormone melatonin regulates seasonal aggression in Siberian hamsters indirectly via steroid hormones (reviewed in Munley et al., 2018; Munley et al., 2022b); the role of neural steroidogenesis and steroid receptors in mediating the actions of melatonin on aggressive behavior, however, are relatively understudied. Here, we investigated how melatonin regulates seasonal plasticity in neural steroid sensitivity and territorial aggression in male and female hamsters using qPCR. We demonstrated that males and females exhibiting a SD-like melatonin signal, either via timed melatonin injections or SDs, displayed increased aggression and showed similar changes in neural gene expression in the ARC. Interestingly, we also determined that melatonin alters neural steroid sensitivity in a sex-specific manner, as LD-M and SD males and females showed distinct changes in relative *cyp19a1*, *esr1*, and *srd5a3* expression in the MPOA, AH, and PAG. Lastly, we determined via correlation analyses that male and female hamsters show different relationships between aggression and neural gene expression in response to timed melatonin injections and SDs. Taken together, our findings support a growing body of evidence that melatonin induces distinct neuroendocrine responses that may underlie a similar behavioral phenotype (i.e., increased non-breeding aggression) in male and female Siberian hamsters.

4.1. Timed melatonin administration and SDs induce gonadal regression, increase aggression, and produce similar changes in neural gene expression in the ARC of male and female hamsters

As expected, timed melatonin injections and SDs caused characteristic reductions in body and reproductive tissue mass in both male and female hamsters. Interestingly, LD-M and SD females showed similar decreases in body and ovarian mass, whereas SD males showed more pronounced reductions in body and testes mass than LD-M males. Although these results could suggest that the effects of melatonin on body and reproductive tissue mass are more robust in female than in male hamsters, our previous studies generally do not support this finding (Munley et al., 2022c). Moreover, we found that timed melatonin administration and SDs increased aggression in Siberian hamsters, regardless of sex. These results agree with our prior work in Siberian hamsters, in which we have demonstrated that a SD-like melatonin signal increases territorial aggression in male and female hamsters (Munley et al., 2020; Rendon et al., 2015). Surprisingly, treatment with melatonin and SDs induced changes in gene expression in the ARC, including an increase in *cyp19a1* expression and a reduction in *esr1* expression, in both sexes. It is important to note that due to methodological limitations and the small size of the ARC, our microdissected punches may have also contained adjacent brain regions such as the ventromedial and tuberal nuclei of the hypothalamus, the former of which has been implicated in aggression in rodents (Hashikawa et al., 2017; Lee et al., 2014; Yang et al., 2013). Thus, the observed changes in *cyp19a1* and *esr1* gene expression in the ARC could be due, at least in part, to the inclusion of additional nuclei in these samples. The ARC has a well-established role in regulating reproduction via kisspeptin, neurokinin B, and dynorphin neurons, which are critical in conveying the feedback effects of gonadal steroids on the secretion of gonadotropin-releasing hormone, thereby regulating the activity of the hypothalamic-pituitary-gonadal axis (reviewed in Joseph et al., 2013; Kanda, 2019; Lehman et al., 2010). The ARC is also part of a conserved neural circuit that regulates the consummatory aspects of reproductive behavior (i.e., lordosis) in rodents via projections to the MPOA (reviewed in Micevych et al., 2015; Micevych and Meisel, 2017). Because the ARC is associated with reproduction and sexual behavior, but not aggression, we did not expect to see differences in the expression of steroid-related genes in this brain region between treatment groups.

In addition to modulating reproduction and sexual receptivity, however, the ARC has a central role in controlling food intake, energy balance, and feeding behavior via the melanocortin signaling pathway (reviewed in Sohn et al., 2013; Stevenson et al., 2022; Williams and Elmquist, 2012). Notably, prior work suggests that this neural circuit is regulated by melatonin and steroid hormones. Pinealectomy leads to leptin resistance and weight gain in male rats, and male mice lacking the MT_1 melatonin receptor exhibit an increase in the expression of the neuropeptide precursor proopiomelanocortin in the ARC and show deficits in leptin signaling, including increased gain weight and higher food intake after fasting and reductions in leptin-induced phosphorylation in the ARC (Buonfiglio et al., 2018; Fischer et al., 2017; Ríos-Lugo et al., 2015). Moreover, T and E_2 administration increase the mRNA expression of the orexigenic peptide neuropeptide Y (NPY) in the ARC of gonadectomized male and female rats, suggesting that steroid hormones modulate the melanocortin system in the ARC via NPY neurons (Shimizu et al., 1996; Urban et al., 1993). Given the profound endocrine and thermoregulatory changes that Siberian hamsters undergo on a seasonal basis, including gonadal regression and a pronounced reduction in body mass during the non-breeding season, it is possible that the observed changes in relative *cyp19a1* and *esr1* expression in the ARC may be linked to these physiological processes instead of aggressive behavior. In support of this hypothesis, we observed few relationships between aggressive behavior and the expression of steroid-related genes in the ARC in male and female hamsters. Additional studies are necessary to assess whether melatonin mediates seasonal variation in energy balance and feeding behavior via steroids in this species.

4.2. Treatment with melatonin and SDs induces sex-specific changes in neural steroid sensitivity in the MPOA, AH, and PAG

In contrast to the gene expression patterns observed in the ARC, we found marked sex differences in the effects of SD-like melatonin on relative *cyp19a1*, *esr1*, and *srd5a3* expression in the MPOA, AH, and PAG. LD-M and SD males showed a reduction in relative *cyp19a1* expression, but no significant change in relative *esr1* expression in the MPOA, whereas LD-M and SD females exhibited increases in relative *cyp19a1* and *esr1* expression in the MPOA. Conversely, there was no difference in relative *srd5a3* expression in the MPOA in either sex, regardless of treatment. Collectively, these data suggest that treatment with melatonin and SDs *decreases* estrogen production in the MPOA of males, but *increases* estrogen synthesis and sensitivity in the MPOA of females. Our results differ from previous work in female Siberian hamsters, male California mice (*Peromyscus californicus*), male song sparrows, and male spotted antbirds, which showed that the mRNA expression or density of estrogen receptor α and/or estrogen receptor β in the MPOA either does not change or increases following exposure to SDs (Canoine et al., 2007; Rendon et al., 2017; Trainor et al., 2008; Wacker et al., 2010). These discrepancies may be attributed to species-specific seasonal responses and the fact that we measured the expression of the *esr1* gene, whereas prior studies that have examined seasonal variation in ERα and/or ERβ in rodents used immunocytochemistry to measure the abundance of the ERα and/or ERβ protein.

The sex-specific effects of melatonin on *cyp19a1* and *esr1* expression in the MPOA that we observed in the present study are not surprising, given that this brain region is sensitive to steroid hormones and has been shown to be sexually dimorphic with respect to the number and density of neurons, synaptic organization, and gene expression patterns in nearly all vertebrates (reviewed in Freeman and Rissman, 1996; Hull and Dominguez, 2007; Panzica et al., 1996). In addition to controlling aggressive behavior (Albert et al., 1986; Barfield, 1971; reviewed in O'Connell and Hofmann, 2011; Schlinger and Callard, 1989), the MPOA is responsible for regulating sexual and reproductive behaviors, including copulatory behavior in males, lordosis in females, and

parental care in both sexes (Perachio et al., 1979; Riters et al., 1998; Slawski and Buntin, 1995; Takeo et al., 1993; Wei et al., 2018; Wu et al., 2014). Because we found that relative *cyp19a1*, *esr1*, and *srd5a3* expression in the MPOA is correlated with aggressive behavior in a treatment- and sex-specific manner, our findings suggest that these changes in gene expression may contribute to seasonal aggression.

Moreover, we found that timed melatonin injections and SDs increased relative *srd5a3* expression in the AH of male hamsters, but did not affect relative *srd5a3* expression in the AH of female hamsters. Timed melatonin administration and SDs, however, reduced relative *srd5a3* expression in the PAG of females, but did not affect relative *srd5a3* expression in the PAG of males, whereas LD-M and SD males and females both displayed a decrease in relative *cyp19a1* expression in the PAG. Taken together, these results suggest that treatment with melatonin and SDs *increases* androgen metabolism in the AH of male hamsters, but *decreases* androgen metabolism in the PAG of female hamsters and *reduces* estrogen production in the PAG of both males and females. Our findings differ from previously published studies in female Siberian hamsters and male song sparrows, which determined that there is no effect of timed melatonin injections or SDs on the density of aromatase-immunoreactive cells in the PAG of female hamsters (Rendon et al., 2020) and that there is no difference in 5α-reductase activity in the diencephalon (contains the AH) between breeding and non-breeding male sparrows (Soma et al., 2003). These disparities in results may be due to differences in techniques (i.e., measuring protein abundance or enzymatic activity versus mRNA expression) or isoform specificity. For example, we quantified *srd5a3* mRNA expression, whereas Soma et al. (2003) measured the enzymatic activity of all isoforms of 5α-reductase.

To date, few studies have investigated the potential role of 5α-reductase in modulating aggressive behavior, particularly within a seasonal framework. There is considerable evidence, however, that both the AH and PAG are sensitive to androgens. Prior work in rats has shown that the MPOA/AH has a high rate of *in vitro* 5α-reductase activity compared to other brain regions in the hypothalamus and limbic system and that the PAG contains large populations of androgen receptor and ERα immunoreactive neurons (Hamson et al., 2004; Murphy et al., 1999; Selmanoff et al., 1977). In addition, recent studies have determined that MT_1 melatonin receptors, which are considered to be primarily responsible for photoperiodic signal transduction (Reppert et al., 1994; Weaver et al., 1996; reviewed in Reppert, 1997), are present in several regions of the hypothalamus and midbrain, including the AH and PAG (Lacoste et al., 2015; reviewed in Ng et al., 2017; Williams et al., 1995). Given the sensitivity of these brain regions to both melatonin and androgens and their prominent role in controlling aggressive behavior in vertebrates (reviewed in Lischinsky and Lin, 2020; Nelson and Trainor, 2007), future studies should characterize how seasonal plasticity in neural 5α-reductase regulates aggressive behavior and determine how melatonin may mediate these mechanisms differently in males and females.

4.3. *Different neuroendocrine responses, similar behavioral phenotype: potential implications for behavioral evolution*

Perhaps one of the most remarkable results of our study is that melatonin and SDs influence the neural expression of steroid-related genes in a sex-specific manner, despite both male and female hamsters displaying increased aggression. These data build on those of our recently published study, in which we demonstrated that LD-M and SD male and female hamsters exhibit distinct seasonal and melatonin-dependent changes in 3β-HSD activity in the adrenal glands and AH (Munley et al., 2022c). While few studies have investigated how the neuroendocrine regulation of seasonal social behaviors may differ between males and females, previous work has shown that circulating steroid concentrations, neural steroid sensitivity, and aggressive behavior vary in a sex-specific manner in breeding songbirds (e.g., Horton et al., 2014; Rosvall et al., 2012; reviewed in Rosvall et al., 2020). Furthermore, sex differences in the neural and molecular mechanisms regulating social behavior have been documented in non-seasonal mammals, including laboratory rodents and humans (reviewed in Choleris et al., 2018; Morrow, 2015). It is important to note, however, that most sex differences in neuroanatomy and physiology that have been investigated are associated with variation in behavior. To our knowledge, this study is among the few to suggest that melatonin has sex-specific effects on neural steroid sensitivity and territorial aggression.

The observed sex differences in the neural expression of steroid-related genes, but not aggressive behavior, raise a critical question: why would male and female hamsters have evolved distinct neuroendocrine responses to melatonin and SDs to regulate a similar behavioral phenotype? Sex differences in the neuroendocrine regulation of behavior may arise through the process of compensation, in which natural selection favors the evolution of distinct mechanisms in males and females to compensate for naturally occurring differences in physiological conditions between the sexes and prevent overt differences in behavior. These compensatory mechanisms are particularly relevant for behaviors displayed by both sexes that are dependent on specific hormonal conditions (e.g., gonadal steroids) or gene expression patterns (e.g., sex chromosomal) that occur in one sex, but never occur in the other (reviewed in De Vries, 2004; McCarthy et al., 2012).

Steroidogenesis provides many avenues through which natural selection can act to produce sex differences in physiology and behavior. Steroid production depends on the regulation of endocrine axes that span multiple tissues, including the hypothalamus, pituitary gland, gonads, and adrenal glands. Within target tissues, there can be also differences in steroid concentrations, the activity of steroid-synthesizing and steroid-metabolizing enzymes, the sensitivity and abundance of steroid receptors, and receptor- or non-receptor mediated secondary messenger systems (reviewed in Fuxjager and Schuppe, 2018; Rosvall, 2022). Moreover, at the molecular level, the differential expression of genes (e.g., steroid-related genes or genomic targets of steroid receptors) can enable individuals to fine-tune physiological mechanisms to reach an optimal behavioral phenotype that is favored by natural selection (reviewed in Ellegren and Parsch, 2007). Together, the complex systems that facilitate the production and mechanisms of action of steroid hormones provide numerous pathways through which selection can act to promote the evolution of specific physiological and behavioral phenotypes, which can vary by sex and even among individuals. The findings of the current study suggest that seasonal aggression in Siberian hamsters is an example of sexual convergence, in which males and females exhibit marked differences in neuroendocrine mechanisms that converge at the same behavioral endpoint: increased non-breeding aggression. Although further research is necessary to elucidate the extent of these sex differences in neural gene expression in male and female hamsters and to determine how these distinct responses may have evolved, our results underscore the importance of including both sexes when studying the neuroendocrine regulation of social behavior, even when males and females display a similar behavior.

5. Conclusions

In the current study, we showed that Siberian hamsters exhibit marked seasonal variation in the neural expression of steroid related genes (i.e., *cyp19a1*, *esr1*, and *srd5a3*), which is dependent on the pineal hormone melatonin. Moreover, we demonstrated pronounced sex-specific effects of timed melatonin administration and SDs on neural steroid sensitivity in brain regions associated with aggression, including the MPOA, AH, and PAG. Future research should characterize the functional implications of these sex-specific effects of melatonin on neural gene expression and to determine how these sex differences in physiological responses may have evolved in male and female hamsters. Collectively, these findings enhance our understanding of how melatonin controls seasonal aggression indirectly via steroid hormones in

seasonally breeding mammals. More broadly, our study provides insight into the processes by which natural selection can, and has, acted on the neuroendocrine mechanisms underlying seasonal social behaviors in a sex-specific manner.

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CRediT authorship contribution statement

K.M.M. and G.E.D. designed the experiments. K.M.M. performed photoperiodic treatments, administered timed melatonin injections, staged behavioral interactions, performed necropsies, collected tissues, determined seasonal phenotypes, scored behavioral videos, sectioned and microdissected brain tissue, performed neural histology and imaging, performed cDNA synthesis, analyzed the qPCR data, and conducted statistical analyses. S.M.S. performed RNA extraction. D.M.S. developed and validated the qPCR primer sets, optimized qPCR conditions, and performed qPCR. K.M.M. and G.E.D. interpreted the data and drafted the manuscript, with editorial contributions from D.M.S. and S.M.S.

Declaration of competing interest

The authors have no competing interests to disclose, financial or otherwise.

Data availability

Data for this study are available in Mendeley Data.

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Appendix A. Supplementary data

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