Photoperiod reverses the effects of estrogens on male aggression via genomic and nongenomic pathways


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Notes:
Photoperiod reverses the effects of estrogens on male aggression via genomic and nongenomic pathways

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Despite recent discoveries of the specific contributions of genes to behavior, the molecular mechanisms mediating contributions of the environment are understudied. We demonstrate that the behavioral effects of estrogens on aggression are completely reversed by a discrete environmental signal, day length. Selective activation of either estrogen receptor α or β decreases aggression in long days and increases aggression in short days. In the bed nucleus of the stria terminalis, one of several nuclei in a neural circuit that controls aggression, estrogen-dependent gene expression is increased in long days but not in short days, suggesting that estrogens decrease aggression by driving estrogen-dependent gene expression. Estradiol injections increased aggression within 15 min in short days but not in long days, suggesting that estrogens increase aggression in short days primarily via nongenomic pathways. These data demonstrate that the environment can dictate how hormones affect a complex behavior by altering the molecular pathways targeted by steroid receptors.

Estrogen receptor | Social behavior | Peromyscus polionotus | Seasonality

Genes code for the molecular machinery that interacts with the environment to regulate behavior. Despite the importance of gene–environment interactions, relatively few studies have explored the mechanistic bases of these processes (1). These interactions may generate apparent inconsistencies in relationships between neurochemical systems and behavior (2). For example, in most birds and domesticated mice estrogens increase aggression, whereas estrogens decrease aggression or its components in Bluebanded gobies, California mice, and humans (3). This complexity in estrogenic modulation of aggression could be mediated by several factors including differential expression of estrogen receptor (ER) subtypes or differences in receptor activity after estrogen binding. In male vertebrates estrogens can be produced in the testes or synthesized in the brain from androgens. ERs can modulate physiology and behavior via both genomic and nongenomic pathways (4). Estrogens can alter the transcription of other genes by translocating to the nucleus and binding to estrogen response elements (ERE) or other response elements (5), a process mediated by an array of cofactors (6). Estrogens can also exert a variety of nongenomic effects that may be mediated by unique membrane-bound receptors (7) or the well characterized ERα (8) and ERβ (9). Recent studies suggest ERα and ERβ can be located at the membrane (10) and may facilitate phosphorylation of MAP kinase and CREB (11). Although the transduction of estrogenic signals has been studied intensively, comparatively little is known about how the environment affects estrogen action.

Using a discrete environmental signal, day length (photoperiod), we have discovered a striking gene–environment interaction. Similar to other rodents, male beach mice (Peromyscus polionotus) exhibit testicular regression and are more aggressive when housed in winter-like short days (12–14). In hypothalamic and limbic brain areas, ERα expression was increased in short days, whereas ERβ expression was increased in long days (53).

Studies of aggression (15–17) and anxiety (18) have suggested that ERα and ERβ can have opposing effects on behavior, and we tested whether photoperiod regulation of receptor expression mediated the effect of photoperiod on aggression. Both ERα and ERβ selective agonists increased aggression in short days but decreased aggression in long days, indicating that simple changes in receptor expression could not explain the effect of photoperiod on estrogen-mediated aggression. The results of a microarray study indicated that estrogen-dependent gene expression in the bed nucleus of the stria terminalis (BNST) was increased in long-day mice compared with short-day mice. Together with the lateral septum, anterior hypothalamus, and medial amygdala, the BNST is part of a neural circuit that mediates a variety of social behaviors including aggression (19, 20). We hypothesized that the apparent reduction in estrogen-dependent gene expression in short-day mice reflected nongenomic action of estrogens in short days. We then observed that fadrozole acts rapidly to increase aggression in short days (consistent with nongenomic activation) but not long days (consistent with genomic activation). Collectively, these data illustrate that the environment determines the effects of estrogens on aggression in Peromyscus and outline a mechanistic basis that could be important in other estrogen-dependent processes.

Results

Photoperiod Determines the Directional Effects of ERα and ERβ Activation. To test whether photoperiod regulation of ER subtype expression causes changes in aggressive behavior, we conducted a hormone manipulation experiment. Males housed in long days or short days for 8 weeks were castrated and fitted with implants to normalize testosterone. To test whether the effect of estrogens on aggression depends on photoperiod, males were treated with fadrozole (an estrogen synthesis inhibitor). In long days, fadrozole increased attacks (Fig. 1A) and decreased attack latency (Fig. 1C) versus vehicle, consistent with a previous report in Peromyscus (21). In short days, fadrozole reduced offensive attacks (Fig. 1B) and increased attack latency (Fig. 1D) versus vehicle.}

Author contributions: B.C.T. and R.J.N. designed research; B.C.T., M.S.F., and M.R.R. performed research; B.C.T. and S.L. analyzed data; and B.C.T. and R.J.N. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: BNST, bed nucleus of the stria terminalis; PPT, propyl pyrazole triol; DPN, diarylpropionitrile; ER, estrogen receptor; MPOA, medial preoptic area; ERE, estrogen response element; cE2, cyclodextrin-conjugated estradiol.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5795).

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Next, we tested whether concurrent treatment with estradiol could block the effects of fadrozole. Estradiol prevented the effects of fadrozole on offensive attacks (Fig. 1A and B) and attack latency (Fig. 1C and D) both in long and short days. This effect cannot be explained by differential metabolism of estrogens by the liver, because there was no effect of photoperiod on plasma estradiol concentrations [see supporting information (SI) Fig. 4]. These data show that estrogens directly regulate aggression and that the direction of the effect depends on photoperiod.

To test whether behavioral effects of estrogens occur via subtype-specific ER activation, we treated animals with fadrozole and ER subtype selective agonists. To examine the effect of ERα, animals were given fadrozole and either a low dose (1.0 mg/kg) or a high dose (4.5 mg/kg) of propyl pyrazole triol (PPT). This agonist has a 400-fold higher affinity for ERα than for ERβ (22). At the higher dose, PPT counteracted the effect of fadrozole on offensive attacks in both long and short days (Fig. 1A and B). The higher dose of PPT blocked the effect of fadrozole on attack latency in long days (Fig. 1C) but not in short days (Fig. 1D). To examine the effect of ERβ animals were treated with fadrozole and diarylpropionitrile (DPN) (10 mg/kg), which has a 70-fold higher affinity for ERβ (23). Treatment with DPN blocked the effect of fadrozole on offensive attacks (Fig. 1A and B) and attack latency (Fig. 1C and D) in both long and short days.
To determine whether photoperiod regulation of estrogen action affected other behaviors besides aggression, we examined the effects of fadrozole and DPN on anxiety-like behavior. Compared with vehicle-treated males, males treated with fadrozole spent significantly less time in the open arms of the elevated plus maze and made fewer entries into open arms, regardless of photoperiod (SI Fig. 5). As has been reported in female rats (24, 25), DPN had significant anxiolytic effects, increasing time spent in the open arms and the number of entries in both long and short days. Thus, not all estrogen-sensitive behaviors are affected by an interaction between estrogens and photoperiod.

To summarize, instead of ERα activation increasing aggression and ERβ activation decreasing aggression, we observed that the subtype selective agonists PPT and DPN had very similar effects on resident–intruder aggression. Both agonists increased aggression in short days and decreased aggression in long days. None of these genes were differentially expressed in the MPOA, suggesting that the effect of photoperiod on ERE-regulated gene expression is anatomically specific. An additional possibility is that estrogen-dependent gene expression in Peromyscus MPOA is regulated by other response elements.

Hypothesis Generation Using Microarray Analyses of Gene Expression.

To generate hypotheses to explain the underlying mechanisms of the photoperiod–estrogen interaction, we conducted a microarray study on brain tissue from long-day and short-day Peromyscus. Replicate micropunch samples (long day, n = 4; short day, n = 2) containing the BNST were collected for RNA extraction. Additional punch samples containing the medial preoptic area (MPOA) were collected from the same animals. For each sample, RNA was amplified and hybridized on Affymetrix 420A arrays. Peromyscus and Mus shared a common ancestor ~25 million years before present, which raises the issue of sequence divergence affecting hybridizations. Although this may impair the detection of important genes, sequence divergence should not result in misclassification of genes regulated by photoperiod (false positives). Indeed, several recent studies have successfully used heterologous hybridizations (26, 27) to study gene expression in “non-model species.”

We focused our analyses on genes that were identified as regulated by EREs via ChIP promoter microarray analysis (ChIP-chip) (28, 29). We next identified genes that showed at least a 1.3-fold difference between long days and short days (n = 8) (Table 1). After conducting independent t tests for each gene, we controlled for the false discovery rate (30). Using a false discovery rate of 5%, a total of 11 genes were classified as differentially expressed, of which 9 were up-regulated in long days. None of these genes were differentially expressed in the MPOA, suggesting that the effect of photoperiod on ERE-regulated gene expression is anatomically specific. An additional possibility is that estrogen-dependent gene expression in Peromyscus MPOA is regulated by other response elements. These data suggest that the expression of ERE-regulated genes in the BNST is either increased in long days or suppressed in short days.

To follow up these results, we conducted real-time PCR for XRCC1 in punch samples of the BNST and MPOA. XRCC1 is an estrogen-dependent transcript (28, 29), and initial RT-PCR experiments showed that this transcript was highly abundant in beach mouse brain punch samples. We measured XRCC1 as a marker of estrogen-dependent gene expression, although it is not yet clear how this gene affects behavior. We examined long-day and short-day animals treated with either saline or fadrozole (n = 4 per group). In saline-treated animals, XRCC1 expression was significantly increased in long days compared with short days (Fig. 2A). Fadrozole treatment decreased XRCC1 expression in long days but not short days (Fig. 2A). Neither photoperiod nor fadrozole treatment affected XRCC1 expression in the MPOA.
(Fig. 2B). Consistent with the microarray data, patterns of XRCC1 expression suggest that in the BNST, ERE-regulated genes are either increased in long days or suppressed in short days. Previous studies on the social behavior network suggest that the role of the MPOA in the context of aggression is minimal compared with other nuclei such as the BNST, medial amygdala, and anterior hypothalamus (20). In Mus musculus, the number of ERα immunoreactive cells is positively correlated with male aggression in the BNST, lateral septum, and anterior hypothalamus, but not in the MPOA (31). Further studies are needed to identify the specific nuclei that mediate the effects of estrogens on aggression in Peromyscus. To explain how estrogens may increase aggression in the apparent absence of estrogen-driven gene expression, we hypothesized that in short days estrogens act primarily via nongenomic pathways.

**Test of the Hypothesis of Environmentally Mediated Nongenomic Estrogen Action.** To test whether estrogens act via nongenomic pathways in short-day mice, we examined whether a s.c. injection of cyclodextrin-conjugated estradiol (cE2) could affect aggressive behavior within 15 min. The genomic effects of estrogens generally occur on a time scale of hours or days, whereas nongenomic effects of estrogens can occur within minutes or seconds (32). Peripheral injection of water-soluble cE2 results in rapid activation of estrogen-dependent reproductive behavior (33, 34), indirect markers of brain activity (35), and MAP kinase and CREB via phosphorylation (11). If estrogens reduce aggression via genomic mechanisms, then no effect of cE2 injection on long-day mice should be observed within 15 min. Likewise, if estrogens increase aggression via nongenomic mechanisms in short-day mice, then cE2 injections should increase aggression in short-day mice within 15 min.

*P. polionotus* housed in either long days or short days were treated with the aromatase inhibitor fadrozole for 6 days. Fifteen minutes before each resident–intruder test, each male was injected with either saline or 75 μg/kg cE2 dissolved in saline. Injections of cE2 increased offensive attacks relative to saline injections in short days but not long days (Fig. 3A). A similar but nonsignificant pattern was observed for boxing behavior (P = 0.06) (Fig. 3C), whereas there were no significant effects of injection photoperiod, or the interaction for attack latency (Fig. 3B). These data suggest that estrogens act via nongenomic mechanisms to increase offensive attacks in short-day mice and that these mechanisms are less active in long-day mice.

**Discussion**

Our studies show that a single environmental factor markedly changes the behavioral action of estrogens. In long days treatment with estradiol or selective ER agonists decreased aggression, whereas in short days these same treatments increased aggression. These data suggest that the basis for this interaction may not depend on simple changes in nuclear ER expression level in the BNST or medial amygdala, because selective activation of either ERα or ERβ yielded similar behavioral responses. Using microarrays and real-time PCR, we observed that expression of estrogen-dependent genes was generally increased in long days versus short days in the BNST. Based on these results we hypothesized that in short-day mice estrogens act primarily via nongenomic pathways to increase aggression. This hypothesis was supported by observations that estradiol acts rapidly to increase aggression in short-day mice but has no rapid effect in long-day mice. These data provide a mechanistic explanation for a complex interaction among photoperiod, estrogen action, and aggressive behavior.

**Nongenomic Pathways of Estrogen Action.** The rapid effect of cE2 injections on offensive attacks in short-day mice supports the hypothesis that estrogens increase aggression via nongenomic pathways in short days. Fifteen minutes is generally considered to be insufficient time for genomic actions of estradiol to occur. Although previous studies have shown that estrogens can affect reproductive behaviors via genomic (36, 37) and nongenomic (4, 33, 34) pathways, to our knowledge this is the first demonstration that the environment can reverse the behavioral action of a steroid by determining which class of molecular pathways is activated. Estradiol can increase the excitability of neurons in the hypothalamus, medial amygdala, and hippocampus within minutes (38). Possible mechanisms for these rapid effects include activation of the cAMP cascade (39) or phosphorylation of protein kinase A (40) and MAP kinase (41). Estrogens can also exert nongenomic effects by regulating calcium channels (42).

Currently there is some uncertainty as to which receptors mediate nongenomic effects of estrogens. The existence of a distinct membrane ER was suggested by observations that estradiol induced phosphorylation of MAP kinase in neocortical explants from ERα knockout mice (41) and subsequent isolation
of a putatively novel ER classified as ER-X (7). More recent evidence suggests that the classical ERs can also act nongenomically. Acute estradiol injection increased phosphorylation of CREB in the medial preoptic nucleus of ERα and ERβ knockout mice, but not ERβ knockout mice (11). Thus, in addition to possible distinct membrane receptors, it appears that either ERα or ERβ can induce nongenomic effects, possibly by undergoing conformational structural changes at the membrane (43). Our results are consistent with nongenomic action of ERα and ERβ, but further study is needed to directly test whether these receptors have nongenomic effects in Peromyscus or whether nongenomic effects are mediated by distinct membrane receptors.

Possible Genomic Pathways of Estrogen Action. In long-day mice, treatment with estradiol inhibited aggression when applied over a 12-day experiment, but not 15 min after injection. We also showed that long-day mice had increased estrogen-dependent gene expression in the BNST. Together, these data suggest that estrogens decrease aggression in long-day mice via slower acting processes, possibly changes in gene expression. It is not yet clear whether the specific genes identified in our microarray experiment play a direct role in mediating the inhibitory effect of estrogens on aggression. Given the relatively low power of microarray experiments, it will be necessary to use a more targeted approach to identify the molecular pathways regulating aggression in long-day mice. Previous studies on estrogen-dependent behavior have identified promising candidate genes. For example, estrogenic regulation of oxytocin signaling is thought to mediate the effects of estrogens on social recognition (44), which could affect aggressive behavior (45). Application of ChIP to identify genes bound by ERα in Peromyscus brain samples could be an effective systematic approach to identifying additional pathways that are not yet implicated in the control of social behaviors. This would also allow for investigation of estrogen-dependent genes that are regulated by other response elements such as AP-1, SP-1, and NfκB (5, 46). It will also be important to investigate the role of ERβ-mediated transcription. Although ERβ has a similar DNA binding domain as ERα, its transcriptional effects await more extensive characterization.

Environmental Regulation of Estrogen Action. Our results demonstrate that in adult Peromyscus the effect of ERα or ERβ on aggression depends on photoperiod and that selective activation of either receptor subtype can increase or decrease aggression in a resident–intruder test. These findings add a new dimension to our understanding of the estrogenic regulation of aggressive behavior. Although most studies report that estrogens increase male aggression, other studies report that estrogens decrease aggression (3). Our results suggest that this diversity is a real biological phenomenon and that future studies conducted under different environmental conditions could reveal additional environment-dependent variation in the estrogenic regulation of behavior. There is also evidence that the environment may affect the behavioral effects of estrogens in other contexts. Although we observed that photoperiod did not influence the effect of DPN in the elevated plus maze, other studies have demonstrated that the effects of estrogens on learning and memory can depend on affective state (18). Our data suggest that it will be worthwhile to investigate whether emotional arousal modulates the molecular actions of estrogens. Thus, in addition to studying receptor expression, it is critical to understand the processes that occur subsequent to receptor activation (4). Given the importance of estrogen signaling in other physiological processes such as cellular proliferation (47) and memory (48), the environmental regulation of estrogenic signaling could be of broad importance.

Methods

Animals. P. polionotus were purchased from the Peromyscus Stock Center (Columbia, SC). Mice at the Stock Center are bred in long days (16-h light:8-h darkness). On arrival at our laboratory, all males were individually housed and randomly assigned to be kept in long days (16-h light:8-h darkness) or short days (8-h light:16-h darkness). Animals were given free access to phytoestrogen-free food (2016; Harlan Teklad, Madison, WI) and filtered tap water ad libitum. All procedures were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Resident–Intruder Tests. For each aggression test, a group-housed sexually inexperienced male was introduced into each resident’s home cage for 10 min under dim red light (1500–1700 hours). When scoring videotaped observations, we defined the number of offensive attacks as the number of bites directed toward the flanks of the intruder. To reduce the effects of some extreme observations, offensive attacks and attack latencies were square-root-transformed for statistical analysis.

Hormone Manipulation and Measurement. Male P. polionotus were housed in either long or short days for 8 weeks before undergoing hormone manipulation surgery. All males were anesthetized with isoflurane and castrated with a single incision through the scrotum. All males were implanted s.c. with a 10-mm silastic implant (i.d. 1.47 mm, o.d. 1.96 mm) packed with 2 mm of crystalline testosterone. Males treated with vehicle received osmotic minipumps (model 2002; Alzet, Palo Alto, CA) filled with 50% Dulbecco’s PBS (dpBS) and 50% DMSO. Males treated with the estrogen synthesis inhibitor fadrozole (0.25 mg/kg) received Alzet minipumps filled with 50% fadrozole dissolved in dpBS and 50% DMSO. Fadrozole is known to affect estrogen-dependent aggression (21) in Peromyscus. Males treated with 17β-estradiol received an additional Silastic implant packed with 1 mm of crystalline 17β-estradiol (i.d. 1.47 mm, o.d. 1.96 mm). Although this dose produced supraphysiological levels of plasma estradiol, the concentration of locally produced estradiol in the brain can be much higher than plasma. Males treated with the ERα agonist PPT received either a low dose (1 mg/kg) or a high dose (4.5 mg/kg) dissolved in DMSO (22). Males treated with the ERβ agonist DPN received 10 mg/kg dissolved in DMSO (49). Minipumps were filled with 50% fadrozole in dpBS and PPT, DPN, or DMSO vehicle. After surgery all males were treated with buprenorphine (0.38 mg/kg). After 12 days, males were tested in resident–intruder tests. The following morning males were anesthetized with isoflurane and rapidly decapitated. Plasma samples were collected, and estradiol was measured by using a direct RIA (DSL-4800; Diagnostic Systems Laboratories, Webster, TX). Plasma samples from intact long-day (n = 6) and short-day (n = 6) males from a different study were run for reference. The intrassay coefficient of variation was 14.4%

Microarray Study. Replicate 1-mm micropunch samples (long day, n = 3; short day, n = 2) containing the BNST were collected for RNA extraction as described above. For each sample, 30 ng of RNA was amplified and hybridized on an Affymetrix 420A array at the Functional Genomics Core at Columbus Children’s Hospital (Columbus, OH). Probe-level data were background-adjusted, normalized, and summarized by using the three-step Robust Multichip Average method (50, 51). We examined genes known to be driven by estrogens and contain EREs in their promoters (28, 29). Because these studies were conducted on human tissue, we used the DRAGON ERE finder (52) to confirm that these genes have EREs upstream of the transcription site in Mus. We further refined these criteria by selecting
genes that showed a 1.3-fold difference between long days and short days (n = 28). Finally, for each gene we compared long-day and short-day expression using t tests. From the raw P values, we calculated adjusted P values to correct for multiple testing using the false discovery rate method (30).

**Quantitative Real-Time PCR.** For measurements of *XRC1 C* (Genbank accession no. EF026105) mRNA, punch samples containing the BNST or MPOA were collected from *P. polionotus* that were housed in long days or short days for 8 weeks and treated with fadrozole (to R.J.N.). For each sample, 1 μg of RNA was reverse transcribed with Moloney murine leukemia virus (Invitrogen). We designed specific primers and probes (see SI Methods) for amplification of *XRC1 C* using the TaqMan system (Applied Biosystems, Foster City, CA). Relative gene expression of a duplicate individual samples was calculated by comparison to a relative standard curve followed by normalization to 18S rRNA gene expression.

**Rapid Effects of Estrogen.** Male *P. polionotus* were housed in either long days or short days for 8 weeks. All males were implanted with Alzet osmotic pumps (model 1007D) containing fadrozole (0.25 mg/kg) dissolved in saline. One week after surgery all males were randomly assigned to receive a s.c. injection of either saline or E2 (75 μg/kg) dissolved in saline. Injections of water-soluble E2 result in a rapid increase in plasma estradiol and have been used by several investigators to examine the effects of estrogens on behavior (33, 34) and brain function (11, 35) on a short time scale. Fifteen minutes after injection males were tested in resident–intruder aggression tests. It is generally agreed that changes in behavior or brain function observed within this time frame are not consistent with a genomic mechanism (32).

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