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Non-invasive administration of 17β-estradiol rapidly increases aggressive behavior in non-breeding, but not breeding, male song sparrows

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ABSTRACT

17β-Estradiol (**E_2**) acts in the brain via genomic and non-genomic mechanisms to influence physiology and behavior. There is seasonal plasticity in the mechanisms by which E_2 activates aggression, and non-genomic mechanisms appear to predominate during the non-breeding season. Male song sparrows (*Melospiza melodia*) display E_2-dependent territorial aggression throughout the year. Field studies show that song sparrow aggression *during* a territorial intrusion is similar in the non-breeding and breeding seasons, but aggression *after* an intrusion ends differs seasonally. Non-breeding males stop behaving aggressively within minutes whereas breeding males remain aggressive for hours. We hypothesize that this seasonal plasticity in the persistence of aggression relates to seasonal plasticity in E_2 signaling. We used a non-invasive route of E_2 administration to compare the non-genomic (within 20 min) effects of E_2 on aggressive behavior in captive non-breeding and breeding season males. E_2 rapidly increased barrier contacts (attacks) during an intrusion by 173% in non-breeding season males only. Given that these effects were observed within 20 min of E_2 administration, they likely occurred via a non-genomic mechanism of action. The present data, taken together with past work, suggest that environmental cues associated with the non-breeding season influence the molecular mechanisms through which E_2 influences behavior. In song sparrows, transient expression of aggressive behavior during the non-breeding season is highly adaptive: it minimizes energy expenditure and maximizes the amount of time available for foraging. In all, these data suggest the intriguing possibility that aggression in the non-breeding season may be activated by a non-genomic E_2 mechanism due to the fitness benefits associated with rapid and transient expression of aggression.

Introduction

17β-estradiol (**E_2**) acts in the brain via both genomic and non-genomic signaling mechanisms to influence physiology and behavior (Vasudevan and Pfaff, 2008). In the genomic model of steroid action, E_2 binds to cytosolic estrogen receptors (ERs), and the hormone-receptor complex translocates to the cell nucleus, and binds to estrogen response elements in the DNA to alter gene expression (Jensen et al., 1968; McCarthy, 2009; Vasudevan and Pfaff, 2008). These effects generally take several hours or days to develop (Zangenehpour and Chaudhuri, 2002), and lead to persistent changes in physiology and behavior (McCarthy, 2009; McEwen, 2001). However, E_2 also acts on a timescale that is too short to be attributed to changes in gene transcription (Cornil and Charlier, 2010). In this non-genomic model of E_2 action, E_2 binds to plasma membrane-associated ERs, which activate signal transduction cascades including mobilization of cytosolic calcium and phosphorylation of cAMP response element binding (CREB) and mitogen-activated protein kinase (MAPK) (Ivanova et al., 2002; Kelly et al., 1999; Singer et al., 1999). These rapid, non-genomic effects typically occur within minutes and lead to more transient changes in physiology and behavior (Laredo et al., 2014).

Recent data suggest that the signaling mechanisms by which E_2 regulates aggressive behavior are modulated by photoperiod. Specifically, in *Peromyscus* mice, acute E_2 administration rapidly alters aggressive behavior in male subjects housed on short (non-breeding season-like) photoperiods but not those housed on long (breeding season-like) photoperiods (Trainor et al., 2007a, 2008). Further, microarray and real-time PCR analyses indicate that estrogen response element-dependent

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gene expression is higher in animals housed on long photoperiods as compared to those housed on short photoperiods (Trainor et al., 2007a). Taken together, these data indicate that the non-genomic effects of E_2 on aggressive behavior may be more prominent during the non-breeding season, whereas the genomic effects of E_2 on aggression may be more prominent during the breeding season (Trainor et al., 2007a).

Like *Peromyscus*, male song sparrows (*Melospiza melodia*) display E_2-dependent territorial aggression throughout the year (except for a brief period during molt) (Wingfield and Soma, 2002). Territorial aggression in song sparrows is measured in the field via simulated territorial intrusion (**STI**) whereby a live caged decoy and conspecific song playback are used to elicit aggressive behavior in residents (Soma et al., 2000). Territorial aggression *during* a STI is both qualitatively and quantitatively similar in the breeding and non-breeding seasons (Wingfield and Hahn, 1994). However, territorial aggression *after* a STI is terminated changes seasonally. In the breeding season, residents continue patrolling their territories and exhibit spontaneous song for hours (even days) after a STI, whereas in the non-breeding season, residents stop behaving aggressively within minutes (Wingfield, 1994). It appears that once the behavior is elicited, breeding territorial aggression is persistent, whereas non-breeding territorial aggression is transient.

Acute inhibition of E_2 synthesis significantly inhibits aggressive behavior in male song sparrows in the non-breeding season only (Soma et al., 2000). Further, acute administration of E_2 lowers CREB phosphorylation in the medial preoptic nucleus, a brain area implicated in aggression in songbirds, in the non-breeding season only (Heimovics et al., 2012b). These data, taken together with the *Peromyscus* studies, raise the hypothesis that non-breeding territorial aggression in male song sparrows is transient because it is activated by non-genomic E_2 signaling mechanisms. We test this hypothesis here using a non-invasive route of E_2 administration to compare the rapid (within 20 min) non-genomic effects of E_2 on aggressive behavior in captive male song sparrows during the non-breeding versus breeding season.

Materials and methods

Subjects and housing

In the Pacific Northwest, song sparrows do not migrate, and males defend territories throughout the year (Arcese, 1989; Wingfield and Monk, 1992). Thus, conspecific song playback and mist nets were used to capture free-living adult male song sparrows both in late October/early November (non-breeding season) and in May (breeding season) near Vancouver, British Columbia (49° 12′N, 123° 01′W). After capture, subjects were transported to the University of British Columbia's Animal Care Centre Annex and housed outdoors in individual wire cages (91 cm × 47 cm × 47 cm) where they were exposed to the natural photoperiod (non-breeding season ≈ 10 L:14D; breeding season ≈ 17 L:7D) and temperature (non-breeding season $\bar{x} \approx 3.8$ °C; breeding season $\bar{x} \approx 15$ °C). Each cage contained two wooden perches and conifer branches. Seed and water were provided ad libitum, and one wax moth larva was provided daily. Except for behavioral tests, subjects were visually isolated from one another throughout the study. Subjects habituated to captivity for 4–6 weeks prior to the onset of behavioral testing. Breeding season males were in reproductive condition throughout behavioral testing as evidenced by the fact that their gonads were recrudesced and plasma T levels were elevated upon euthanasia one month after the present study was complete (Heimovics et al. *in prep.*). Similarly, non-breeding season males were in non-breeding condition throughout behavioral testing as evidenced by the fact that their gonads were regressed and plasma T levels were basal upon euthanasia one month after the present study was complete (Heimovics et al. *in prep*). Protocols were approved by the University of British Columbia Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

Non-invasive route of E_2 administration

To eliminate stress associated with an injection, we used a non-invasive route of E_2 administration. Specifically, E_2 (or vehicle control [CON]) was injected into wax moth larvae that were subsequently fed to subjects immediately prior to behavioral testing. Orally administered steroid hormones enter into the blood stream very rapidly (Breuner et al., 1998), and this route of administration has been used previously to examine rapid effects of steroid hormones on behavior in birds, fish, and mice (Breuner et al., 1998; Hodgson et al., 2008; Laredo et al., 2013; Remage-Healey and Bass, 2006; Saldanha et al., 2000). We found that if wax moth larvae are provided to song sparrows daily throughout habituation to captivity, they will consume larva within 30 sec of it being placed in their cage. Thus, this provides a consistent, efficient, and reliable route of steroid administration.

The night before each day of testing, larvae were placed in a 4 °C refrigerator in order to render them immobile and cause them to desiccate slightly. The next morning, a Hamilton syringe was used to inject larva with 20 μL of either E_2 or CON solution (for details, see below). The syringe was inserted into the ventral surface of the larva, posterior to the last pair of legs. Solution was injected and then the syringe needle was removed slowly to prevent fluid from escaping the larva. In the event that fluid did escape, the larva was discarded and another prepared. Larvae were prepared no more than 5 min prior to being fed to subjects.

Preliminary studies

Oral E_2 dose

We previously examined seasonal plasticity in the rapid effects of E_2 on the male song sparrow brain (Heimovics et al., 2012b). 15 min after an s. c. injection of 500 μg/kg E_2 (12. 5 μg E_2 per subject), widespread effects on phosphorylated extracellular signal-regulated kinase (pERK), tyrosine hydroxylase (pTH), and cAMP response element binding protein (pCREB) were observed (Heimovics et al., 2012b). A s. c. dose of 500 μg/kg E_2 was selected because this dose, but not lower doses, rapidly modulates sexual behavior in quail (Cornil et al., 2006). Thus, the goal of this preliminary study was to determine an oral dose of E_2 that would achieve a similar circulating level of E_2 as 12.5 μg s. c. (8.3 ± 0.9 ng/mL) (Heimovics et al., 2012b).

Wax moth larvae were injected with either 100, 200, or 400 μg E_2 (Steraloids) dissolved in (2-hydroxylpropyl)-β-cyclodextrin (0. 5 mg/mL in PBS) (Sigma #C0926). These larvae were subsequently fed to five male song sparrows in a randomized, counter-balanced order with at least 72 h washout between doses. 15 min after ingestion, subjects were captured and blood from the brachial vein was collected into microhematocrit tubes. Tubes were stored on wet ice until centrifugation (within 3 h). After centrifugation, plasma was collected and stored at −20 °C. E_2 levels were measured in plasma in duplicate using a commercially available ^{125}I-E_2 radioimmunoassay kit (DSL-4800, Ultra-sensitive Estradiol RIA, Beckman Coulter). Methods for this E_2 RIA have been published extensively elsewhere (Charlier et al., 2010, 2011; Heimovics et al., 2012b; Taves et al., 2010). Mean plasma E_2 levels for each dose was determined, a line of best fit was calculated ($y = 1.1962 + 0.0274x$), and it was concluded that an oral dose of 300 μg E_2 would achieve circulating levels of E_2 comparable to 12.5 μg E_2 s. c. at 15 min after E_2 administration (Fig. 1).

Importantly, the plasma concentration of E_2 achieved here far exceeds plasma levels seen in free-living birds (Soma and Wingfield, 1999b, 2001). However, prior work in songbirds demonstrates E_2 levels in brain punches can be two orders of magnitude higher than in plasma (Charlier et al., 2010, 2011; Taves et al., 2011) and E_2 concentrations at aromatase-positive synapses may be even higher than in punches