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Rapid effects of estradiol on aggression depend on genotype in a species with an estrogen receptor polymorphism

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Abstract

The white-throated sparrow (*Zonotrichia albicollis*) represents a powerful model in behavioral neuroendocrinology because it occurs in two plumage morphs that differ with respect to steroid-dependent social behaviors. Birds of the white-striped (WS) morph engage in more territorial aggression than do birds of the tanstriped (TS) morph, and the TS birds engage in more parenting behavior. This behavioral polymorphism is caused by a chromosomal inversion that has captured many genes, including estrogen receptor alpha (ERα). In this study, we tested the hypothesis that morph differences in aggression might be explained by differential sensitivity to estradiol (E2). We administered E2 non-invasively to non-breeding white-throated sparrows and quantified aggression toward a conspecific 10 min later. E2 administration rapidly increased aggression in WS birds but not TS birds, consistent with our hypothesis that differential sensitivity to E2 may at least partially explain morph differences in aggression. To query the site of E2 action in the brain, we administered E2 and quantified Egr-1 expression in brain regions in which expression of ERα is known to differ between the morphs. E2 treatment decreased Egr-1 immunoreactivity in nucleus taeniae of the amygdala, but this effect did not depend on morph. Overall, our results support a role for differential effects of E2 on aggression in the two morphs, but more research will be needed to determine the neuroanatomical site of action.

1. Introduction

Most, if not all, alternative behavioral phenotypes are linked to variation in sex steroid hormones (Oliveira et al., 2008). Species exhibiting alternative phenotypes therefore represent an opportunity to investigate the mechanisms underlying hormone-dependent behaviors and their evolution. One such species is the white-throated sparrow (*Zonotrichia albicollis*), which exhibits alternative plumage phenotypes associated with a polymorphism in sex steroid-dependent behaviors. White-throated sparrows occur in two plumage morphs, white-striped (WS) or tan-striped (TS). WS birds respond to territorial threats with higher levels of vocal and physical aggression than do TS birds, which invest more in provisioning nestlings (Falls, 1969; Horton et al., 2014a; Lowther, 1961; Tuttle, 2003; Tuttle et al., 2016).

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This behavioral polymorphism segregates with the presence or absence of a rearrangement on chromosome 2 called ZAL2^{m} (Thomas et al., 2008; Thorneycroft, 1966). All birds with the ZAL2^{m} arrangement exhibit the WS plumage coloration, whereas TS birds lack the rearrangement, having two copies of the standard ZAL2. Suppression of recombination between ZAL2 and ZAL2^{m} has resulted in the divergence of genes inside the rearrangement (Davis et al., 2011; Thomas et al., 2008) such that the two haplotypes are now 1% different from each other (Huynh et al., 2011). Only the WS birds carry a copy of ZAL2^{m}; the genes captured by the rearrangement are thus likely responsible for the majority of morph differences in this species, including those in territorial aggression and parenting behavior.

In songbirds, territorial aggression and parental provisioning are known to be affected by sex steroids (Goodson et al., 2005; Hegner and Wingfield, 1987; Schlinger and Callard, 1990; Wingfield, 1985; Wingfield et al., 1987). Therefore, some of the top candidate genes that could explain the morph difference in behavior in white-throated sparrows are those in the sex steroid pathway. The gene for estrogen receptor alpha (ERα), known as ESR1, has been captured by the rearrangement (Thomas et al., 2008) and is differentiating between ZAL2 and ZAL2^{m}. This divergence is thought to cause differential expression of the receptor, and in turn, to affect behavior (see Horton et al., 2014b; Maney, 2017; Maney et al., 2015). In CD-1 mice (*Mus musculus*) and dark-eyed juncos (*Junco hyemalis*), individual variation in ERα expression in a number of brain regions predicts agonistic behavior (Rosvall et al., 2012; Trainor et al., 2006). ERα is well-known to mediate aggression in rodents; in male mice, for example, specific ERα agonists increase attacking behavior (Clipperton-Allen et al., 2011), and knocking out ESR1 reduces aggression (Ogawa et al., 1997; Scordalakes and Rissman, 2004). The clear role for ERα in aggression in other vertebrates makes it a top candidate for mediating morph differences in aggression in white-throated sparrows.

In the white-throated sparrow, ERα expression in at least eight brain regions depends on morph (Horton et al., 2014b). WS birds have more ERα mRNA in nucleus taeniae of the amygdala (TnA), the paraventricular nucleus (PVN), and HVC (used as a proper name). In contrast, TS birds have more ERα mRNA in the rostral medial preoptic area (rPOM), anterior hypothalamus, ventromedial hypothalamus (VMH), bed nucleus of the stria terminalis (BSTm), and ventrolateral portion of the caudal lateral septum (Horton et al., 2014b). Expression in TnA and PVN predicts territorial singing even when morph and sex steroids are controlled in regression analyses; in fact, expression in these areas predicts singing better than morph itself (Horton et al., 2014b).

The tight correlation between ERα expression and territorial aggression suggests that morph-dependent behavior may be caused, in part, by differential actions of estradiol (E2). To test this hypothesis, we need to assess the effects of exogenous E2 on behavior. Previously, we showed that when plasma sex steroids are experimentally equalized between the morphs, the morph difference in aggression persists (Maney et al., 2009). Treating non-breeding males with testosterone for seven days increased aggressive responses to song playback more in WS birds than TS birds. In non-breeding females, seven days of E2 treatment increased spontaneous aggressive vocalizations in WS females only. These results suggest that the neural circuits involved in aggression are more sensitive to E2 in WS than TS birds.

Maney et al. (2009) measured behavior at one time point, after seven days of treatment, which did not provide much information about underlying mechanisms. The steroid hormones administered in that study could have acted *via* genomic or nongenomic mechanisms, or both. When E2 acts *via* nongenomic mechanisms, behavior can be affected rapidly, within minutes. Heimovics et al. (2015a) showed that in non-breeding song sparrows (*Melospiza melodia*), E2 treatment rapidly increased aggression within 20 min. The time course of this result suggested a nongenomic mechanism of E2 action. In the present study, we tested whether E2 treatment increases aggression on the same time scale in white-throated sparrows and if so, whether that effect depends on morph. We administered E2 to birds in non-breeding condition, in which endogenous plasma E2 is very low and thus receptors would not be saturated; nonetheless expression of ERα is higher in WS than TS birds in both TnA and PVN (Maney et al., 2015; B. M. Horton, unpublished). Greater behavioral responses to E2 in WS than TS birds would be consistent with the hypothesis that ERα mediates the behavioral polymorphism in this species.

Finding differential effects of systemic E2 treatment on behavior would not indicate the neuroanatomical site of action. Therefore, we mapped induction of the immediate early gene Egr-1 after E2 administration. Our methods were similar to those of Heimovics et al. (2012), who showed that in song sparrows, exogenous E2 rapidly affected the phosphorylation of molecules in the MAPK signaling cascade, ERK and CREB, in TnA and POM, respectively. Both ERK and CREB serve as transcription factors to induce the transcription of many genes, including Egr-1 (Shi et al., 2002). We hypothesized that if morph differences in E2-induced aggression in white-throated sparrows result from differential expression of ERα, then E2 administration should induce differential Egr-1 expression in TnA or other regions with differential expression of ERα.

2. Methods

2.1. Experimental design

In Experiment 1, we tested whether a bolus dose of E2 can rapidly induce aggression, as previously observed in song sparrows (Heimovics et al., 2015a), and if so, whether that effect depends on morph. In Experiment 2, to assess the neural responses to this dose of E2, we administered a dose of E2 identical to that used in Experiment 1 and quantified the expression of Egr-1 in five brain regions in which ERα expression has been shown to depend on morph in this species (Horton et al., 2014b).

2.2. Experiment 1

2.2.1. Animals—All procedures involving animals were approved by the Emory University Institutional Animal Care and Use Committee, were in keeping with all federal, state, and local laws, and adhered to guidelines set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* White-throated sparrows of both sexes and morphs were collected in mist nets on the campus of Emory University in Atlanta, GA during fall migration. Sex was confirmed by PCR analysis of a small blood sample (Griffiths et al., 1998). Assessments of morph were made using a PCR assay (Horton et al., 2013; Michopoulos et al., 2007) and by visual inspection of plumage (Michopoulos et al., 2007;

Piper and Wiley, 1989a). Assessments of age were determined by the shape of the primary coverts and outer rectrices and the degree of skull ossification (Pyle, 1997). Birds were housed in the Emory animal care facility in walk-in flight cages (4′×7′×6′), supplied with *ad libitum* seed and water. The day length was kept constant at 10L:14D, which corresponds to the shortest day the birds would experience on their wintering grounds in Georgia. At least one month prior to behavioral assays, birds were transferred to individual cages (15″×15″ ×17″) inside walk-in sound-attenuating booths (Industrial Acoustics, Bronx, NY). They were housed two to six birds per booth, with opaque barriers between the cages to prevent visual contact, until the behavioral trials. All booths were identical and the day length remained at 10L:14D throughout the experiment.

2.2.2. Pre-screening for social dominance—The goal of Experiment 1 was to determine whether E2 administration affects aggression differently in the two morphs. Like many songbirds, white-throated sparrows establish dominance hierarchies such that subordinate birds rarely show aggression toward dominant ones (Archawaranon et al., 1991). Therefore, because we were interested in measuring the effects of E2 on aggression, we limited our investigation to dominant birds (see Heimovics et al., 2015a). Dominance rank does not depend on plumage morph in non-breeding white-throated sparrows (Archawaranon et al., 1991; Maney and Goodson, 2011; Piper and Wiley, 1989b) and administration of sex steroids does not affect established dominance relationships (Wiley et al., 1993). In order to identify pairs of birds with clear dominance relationships, we performed prescreening trials on same-morph, same-sex pairs. Within these pairs, the dominant bird would become the focal bird and the subordinate the “opponent”. During prescreening trials, the cages of the two birds were placed adjacent to one another in an empty booth. The birds were then allowed to interact vocally and visually for 30 min. The interactions were recorded using a camcorder placed on a tripod ~1 m away so that both cages were completely visible in the video. After the trial, the birds in their cages were returned to the home booth.

To determine which bird in the dyad was dominant, we scored two aggressive behaviors in the videos. Aggression was operationalized according to Heimovics et al. (2015a), who demonstrated a rapid effect of E2 on aggression in a related songbird. First, we quantified attempted attacks, defined as the bird making contact with both feet on the wall of its cage facing the opponent and flapping its wings. We considered this behavior to be clearly aggressive and we never saw a bird perform this behavior on another wall of the cage. Second, every 30 s the observer scored the bird’s position with respect to the opponent’s cage. If the focal bird was located in the third of its cage closest to the opponent, it was considered to be in proximity to the opponent and received a score of “1”. If it was located in the other two-thirds of its cage, in other words not in proximity to the opponent, it received a score of “0”. The zone of the cage defined as proximal to the opponent was marked by, and included, a perch (Fig. 1). The member of the dyad that made more attacks and was more often in proximity to the opponent was deemed dominant. If neither bird in the dyad dominated in terms of both behaviors, the dyad was dissolved and each bird was tested again with a new bird. The dominant bird within each dyad was designated as the focal bird and the subordinate as the opponent for the behavioral trials described below.

2.2.3. Hormone manipulation—In order to minimize any stress associated with delivery of E2, we administered the hormone non-invasively. Non-invasive administration has been used previously to examine the rapid effects of steroids on behavior in songbirds (Breuner et al., 1998; Breuner and Wingfield, 2000; Heimovics et al., 2015a; Hodgson et al., 2008; Saldanha et al., 2000). In male song sparrows, which are in the same family and similar in size (~25 g) to white-throated sparrows, 300 μg of E2 delivered orally induced aggression 10–20 min later (Heimovics et al., 2015a). Therefore, we used the same dose of E2. Larvae of the wax moth (*Achroia grisella*) were prepared as described by Heimovics et al. (2015a). A Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) was used to inject each larva with 20 μl of water containing either 300 μg of cyclodextrin-encapsulated 17β-estradiol (E2; Sigma-Aldrich, cat. no. E4389) or cyclodextrin alone as a control (CON; Sigma-Aldrich, cat no. C0926). In order to habituate the birds to the presentation of the larva, each bird received one larva in a weigh boat on the floor of its cage each day for at least 1 month. After excluding birds that did not consume the larvae within one minute, the final sample size was 1 WS male, 12 WS females, 5 TS males, and 13 TS females. The female bias did not affect our hypothesis because in free-living populations, the morph difference in physical aggression is most pronounced in females (Horton et al., 2014a). A female-biased sample was thus appropriate for our study.

2.2.4. Rapid effects of E2 on behavior—Each bird was treated with E2 and CON in a counterbalanced order. This experimental design allowed us to control for individual differences in aggression at ‘baseline’, as measured during the CON trial. Birds received both treatments in an order that was balanced according to age, sex, and morph, with a 48 h washout period between each trial. We chose this washout period because in song sparrows, plasma E2 is non-detectable 48 h after this dose of E2 (Heimovics et al., 2015a).

Behavioral testing occurred within 3 days of determining which bird was dominant in a dyad (see above). Before a behavioral trial, the focal (dominant) bird in its cage was placed in an empty sound-attenuating booth for 1 h to acclimate it to that environment. One hour later, the opponent in its cage was placed immediately adjacent to the focal bird’s cage with an opaque barrier visually isolating the birds. A larva injected with E2 or CON was placed in a weigh boat on the floor of the focal bird’s cage and the experimenter immediately left the room. The moment when the focal bird consumed the larva was defined as T0 (Fig. 1). Ten min later, at T10, the experimenter quietly entered the booth and removed the opaque barrier, then immediately left. The dyad was then allowed to interact vocally and visually for 10 min (T10–T20; “barrier removed”), during which behavior was recorded using a camcorder placed on a tripod ~1 m away from the cages such that the entire cage of each bird was visible. Ten min later (T20), the experimenter quietly entered the booth and replaced the opaque barrier, then immediately left. Behavior was recorded for ten more min (T20–T30; “barrier replaced”). At T30, the cages were placed back into the home booths.

Behavior between T10 and T30 was scored by an observer blind to morph and treatment. Attacks were scored exactly as described above for the prescreening trials. Time spent near the opponent was defined as the total amount of time during each minute of the ten-min trial that the focal bird spent in the third of its cage closest to the opponent. Although this

definition differed slightly from how we scored behavior during the prescreening trials, it yielded similar results and facilitated data analysis by parametric methods.

As a control for general locomotor activity, the observer also quantified the total number of times the bird alighted, without flapping its wings, on either the wall of its cage facing the opponent or the wall on the opposite side. Unlike the contacts scored as attacks, these contacts occurred on all walls of the cage. In contrast, contacts with wing flapping, which were scored as attacks, occurred only on the wall facing the opponent.

2.2.5. Analysis of behavior—All behavior was analyzed using generalized linear mixed models (GLMM) with negative binomial distributions. Six separate analyses were conducted: one for each behavior (attacks, time spent near opponent, and non-attack wall contacts) in each condition (barrier absent or barrier replaced). For many of the birds, time near the opponent was at the ceiling (60 s per min near the opponent). To accommodate this distribution, we subtracted each value from 60, which produced a zero-inflated dataset for which we could include a zero-inflated correction factor (Zuur et al., 2012). For each test, the best-fit model was selected on the basis of AIC values (Symonds and Moussalli, 2011). When a significant interaction between morph and treatment was detected, post-hoc analyses were conducted to test for an effect of treatment within morph. The factors included in each model are summarized in Supplementary Tables 1, 2. Wald chi-squared tests were used to generate analysis-of-deviance summary tables. As was also reported by Heimovics et al. (2015a), singing occurred too infrequently for statistical analysis. All data were analyzed using the function *glmmADMB* from the glmmADMB package (Fournier et al., 2012; Skaug et al., 2013; v. 0.8.3) in RStudio (RStudio Team, 2015; v. 1.0.136) built on the R software (R Core Team, 2014; v. 3.3.2) and summarized using the function *Anova* from the car package (Fox and Weisberg, 2011). Pseudo-R^2, which is based on the adjusted likelihood ratio (Magee, 1990), was calculated to estimate the effect size for each fixed effect. The α level was set at $p \leq 0.05$.

2.3. Experiment 2

Our goal in Experiment 2 was to determine whether E2 administration has rapid effects on the MAPK pathway and if so, whether that effect depends on morph. As a readout of rapid responses, we measured the protein product of a downstream target of the MAPK pathway, the immediate early gene Egr-1. The expression of Egr-1 protein is known to peak at about 60 min following exposure to a stimulus (Mello and Ribeiro, 1998) and has been used in white-throated sparrows to map neural responses to a variety of pharmacological and sensory manipulations (Maney et al., 2007; Saab et al., 2010; Sanford et al., 2010).

2.3.1. Hormone treatment and tissue collection—All of the birds used in Experiment 1 were used in Experiment 2. For each bird, Experiment 2 began at least one week after Experiment 1 was completed. In order to minimize the possibility that any morph-dependent Egr-1 expression could be caused by morph differences in social behavior, birds were isolated starting two hours before the trial. One hour after being transferred to an empty sound-attenuating booth, each bird was presented with a larva injected with a solution containing 300 μg E2 or vehicle only (CON), the same dose as Experiment 1. Exactly one

hour after consuming the larva, each bird was deeply anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL). Brains were rapidly harvested and fixed in 5% acrolein as previously described (Maney et al., 2003). Ovaries and testes were inspected to verify a regressed state. Blood from the jugular vein was collected into microhematocrit tubes to determine plasma E2 levels at the time of sacrifice (see below).

2.3.2. Histology—Immunohistochemistry was performed on brain tissue as previously described (Maney et al., 2003). Briefly, every third 50-μm section was incubated with a 1:16,000 dilution of polyclonal antibody (anti-Egr-1; cat. #Sc-189, lot #L0104, Santa Cruz Biotechnology, Santa Cruz, CA) raised against the C-terminus of Egr-1 of human origin (sequence STGLSDMTATFSPRTIEIC). This antibody has been used in published investigations of Egr-1-IR (also called ZENK) in the avian brain. Mello and Ribeiro (1998) demonstrated that in zebra finches, incubating the working dilution with a 10-fold excess of Egr-1 peptide results in a complete loss of nuclear staining. In our study, Egr-1 was labeled by using a biotinylated secondary antibody and the ABC method (Vector, Burlingame, CA). Labeling was visualized by using diaminobenzidine enhanced with nickel (Maney et al., 2003; Shu et al., 1988). Sections were mounted onto microscope slides and coverslipped in DPX (Sigma, St. Louis, MO).

2.3.3. Quantification of Egr-1 expression—Because we were primarily interested in responses to E2 that could explain the behavioral polymorphism, we quantified Egr-1-IR in regions where ERα mRNA differs by morph (Horton et al., 2014b). ImageJ software (version 1.44 K; NIH, Bethesda, MD) was used to count the labeled cell nuclei inside a selected area (Supplementary Table 3) within the region of interest (ROI) (Fig. 2). Sections were photographed with a Leica DFC480 camera attached to a Zeiss Axioskop microscope. The 4× objective (total magnification 40×) was used to acquire images. Egr-1-IR was quantified in each bird in 5 ROIs: TnA, PVN, rPOM, VMH, and BSTm. The number of labeled cells was determined as previously described (Maney et al., 2006). Briefly, each image was opened in ImageJ (Bethesda, MD) and the area covered by immunopositive cells, defined by clusters of pixels with an optical density higher than a threshold value, was quantified within each ROI (Fig. 2). Because of variability in background staining among brains, this threshold was set manually for each image such that clusters of pixels highlighted by the computer program agreed with what the observer considered to be labeled nuclei. In order to convert the area measurement to an estimated number of labeled nuclei, the average size of an individual nucleus was determined using an image with few overlapping nuclei (Maney et al., 2003). This average size was then used to estimate the number of labeled nuclei in our images by dividing the total area covered by labeled nuclei by the average size of a nucleus. The estimated number of nuclei was then expressed as cells per unit area. If the tissue inside an ROI was damaged in any one section such that the labeled cells could not be counted, that section was skipped.

2.3.4. Validation of hormone manipulation—To confirm that oral administration of E2 elevated plasma levels on a time course relevant to both Experiment 1 and 2, we collected blood at two time points after administration of E2: 15 min, which corresponds to the middle of the “barrier absent” behavioral trial in Experiment 1, and 60 min, immediately

before harvesting the brains for Experiment 2. For the 15 min time point, six white-throated sparrows (one WS male, two WS females, two TS males, and one TS female) were isolated in sound-attenuating booths for one hour prior to presentation of a wax moth larva injected with 300 μg E2 or CON. All larvae were consumed within 1 min, and each bird remained isolated until the collection of a blood sample from a brachial vein 15 min later. For the 60 min time point, blood was collected from the birds in Experiment 2 immediately prior to sacrifice. For both time points, the microhematocrit tubes containing the blood samples were kept on wet ice and centrifuged to isolate the plasma, which was harvested and stored at −20°C until radioimmunoassay.

2.3.5. Radioimmunoassay—Plasma E2 levels were measured by radioimmunoassay using validated methods (Charlier et al., 2011; Heimovics et al., 2016; Newman et al., 2008; Overk et al., 2013; Taves et al., 2011). Steroids were extracted from plasma samples (20–40 μl per replicate) using solid phase extraction with C18 columns (500 mg, catalog #12113045; Agilent Bond-Elut OH; Agilent Technologies Inc., Santa Clara, CA, USA) as described by Heimovics et al. (2016). Samples were then dried at 40°C in a vacuum centrifuge (ThermoElectron SPD111V Speedvac; Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at −20 °C until assayed.

Dried extracts were resuspended in phosphate-buffered saline containing 0.1% gelatin and 1.0% absolute ethanol. All samples were analyzed in duplicate following the instructions of the manufacturer, with slight modifications in the protocol to increase assay sensitivity (Charlier et al., 2011; Heimovics et al., 2016). 17β-estradiol was measured using a specific and sensitive radioimmunoassay (Beckman Coulter, #DSL-4800, Chino, CA, USA). The detection limit for the E2 assay was 0.20 pg/tube, which corresponded to a concentration of 12.4 pg/ml for most samples.

To calculate steroid recovery, standards of known concentrations (n = 14 total) were processed along with the plasma samples. E2 recovery was 90.7% and sample concentrations were corrected for recovery. As a negative control, water blanks (n = 14 total) were processed along with plasma samples, and none of them contained detectable E2 (all values were below the lowest point on the standard curve, 0.20 pg/tube). Two of the plasma samples were excluded due to apparent contamination.

2.3.6. Analysis of Egr-1 expression—Homogeneity of variance was tested using Levene's test and the distribution of the data was tested using the Shapiro–Wilk test. Then, for each brain region, average Egr-1-IR cell counts per unit area were entered into an ANOVA with morph (WS or TS) and treatment (E2 or CON) as factors. Data were analyzed in RStudio (RStudio Team, 2015; v. 1.0.136) built on the R software (R Core Team, 2014; v. 3.3.2) using the *aov* function of the *stats* package. The α level was set at $p < 0.05$.

2.3.7. Analysis of plasma E2 levels—Homogeneity of variance was tested using Levene's test and the distribution of the data was tested using the Shapiro–Wilk test. Plasma levels of E2 were compared across treatment, morph, and time point using a three-way ANOVA. To verify that the effects of E2 administration on plasma E2 were similar between the morphs, we then tested for an effect of morph at the 60 min time point in E2-treated

birds only using a *t*-test and estimated effect sizes using Cohen's *d* (Cohen, 1992). Effect sizes were expressed as Cohen's *d* for *t*-tests and η^2 for ANOVAs. We did not have enough statistical power to test for morph differences in E2-treated birds at the 15 min time point.

3. Results

3.1. Experiment 1

3.1.1. Aggression with visual barrier absent—Between T10 and T20, when the visual barrier between the focal bird and the opponent was removed, the effect of E2 on both behaviors depended on morph (Fig. 3A, B). There was a significant interaction between treatment and morph for both attacking ($\chi^2 = 5.67$, $p = 0.017$, $R^2 = 0.159$) and time spent near the opponent ($\chi^2 = 5.27$, $p = 0.022$, $R^2 = 0.150$). There was a significant effect of treatment on time spent near the opponent ($\chi^2 = 4.46$, $p = 0.035$, $R^2 = 0.129$), but no other main effects of treatment or morph on either behavior were detected (see Supplementary Tables 1, 2). Post-hoc tests revealed that within WS birds, E2 treatment significantly increased the number of attacks ($\chi^2 = 8.03$, $p = 0.005$, $R^2 = 0.382$) and time spent near the opponent ($\chi^2 = 4.60$, $p < 0.032$, $R^2 = 0.261$). In TS birds, E2 treatment did not increase attacks ($\chi^2 = 0.14$, $p = 0.718$, $R^2 = 0.008$ or time spent near the opponent ($\chi^2 = 0.09$, $p = 0.760$, $R^2 = 0.005$).

3.1.2. Aggression with visual barrier replaced—After the visual barrier was replaced (T20–T30; Fig. 3A, B), the interaction between morph and treatment disappeared for attacking ($\chi^2 = 0.46$, $p = 0.498$, $R^2 = 0.015$) but persisted for time spent near the opponent ($\chi^2 = 8.77$, $p = 0.003$, $R^2 = 0.226$) (Fig. 3B). There were no main effects of treatment or morph on either behavior during this period (Supplementary Tables 1, 2). Post-hoc tests revealed that E2 treatment significantly increased the time that WS birds spent near the opponent ($\chi^2 = 12.60$, $p < 0.001$, $R^2 = 0.492$). There was no effect of E2 treatment on the amount of time TS birds spent near the opponent ($\chi^2 = 0.06$, $p = 0.804$, $R^2 = 0.004$).

3.1.3. General locomotor activity—There was no effect of morph or treatment, nor an interaction between the two, on non-attack contacts with walls either before or after the barrier was replaced (Supplementary Fig. 1). This result suggests that the effect of E2 on aggression in WS birds was not due to an increase in general locomotor activity. The raw data are available as a supplemental file.

3.2. Experiment 2

3.2.1. Egr-1 immunoreactivity—The effects of E2 treatment on Egr-1 expression are summarized in Fig. 4. E2 treatment decreased Egr-1 in TnA, as shown by a main effect of treatment in that region ($F_{1,31} = 6.90$, $p = 0.013$; Cohen's $d = 0.90$). Similarly, E2 treatment tended to reduce Egr-1 expression in rPOM, as shown by a trend for a main effect of treatment in that region ($F_{1,31} = 3.83$ $p = 0.059$; Cohen's $d = 0.68$). No other effects of treatment, morph, or interactions between treatment and morph were significant for any brain region (all $p > 0.25$; Supplementary Table 4).

3.2.2. Plasma E2—Plasma concentrations of E2 are shown in Fig. 5. In both morphs, an oral dose of 300 μg E2 elevated plasma E2 ($F_{1,23}$ =8.50, p =0.008, η^2 = 0.36) to breeding-typical levels (see Horton et al., 2014a). Plasma E2 did not differ between the morphs ($F_{1, 23}$= 0.18, p =0.675, η^2 = 0.01) and there was no interaction between morph and treatment ($F_{1, 23}$ = 0.09, p= 0.769, η^2 < 0.01). Plasma E2 seemed to decrease between 15 and 60 min, but this effect was not significant ($F_{1, 23}$ = 2.15, p = 0.161, η^2 =0.09). There were no interactions between morph and time ($F_{1, 23}$= 0.17, p =0.677, η^2 < 0.01), treatment and time ($F_{1, 23}$ = 0.84, p = 0.368, η^2 =0.04), or morph, treatment, and time ($F_{1, 23}$ = 0.05, p = 0.834, η^2 < 0.01). Within E2-treated birds, there were no obvious differences in plasma E2 between the morphs at 15 min, but we did not have the power to directly test that. Within E2-treated birds at 60 min, there was no effect of morph (t_{15} = 0.44, p = 0.670) and the effect size was small (Cohen's d = 0.22).

4. Discussion

The well-known differences in aggressive behavior between the WS and TS morphs in white-throated sparrows are hypothesized to be mediated by morph differences in sensitivity to E2 (Horton et al., 2014b; Maney et al., 2009). In this study, we tested this hypothesis by manipulating E2 levels and quantifying both the short-term behavioral and neural responses. We found that a large bolus dose of E2 rapidly increased aggressive behavior in WS but not TS birds. This result is consistent with the hypothesis that the neural circuits involved in aggression are more sensitive to E2 in WS than TS birds. The effects of E2 treatment on Egr-1 expression in the brain did not depend on morph, however, suggesting that the mechanisms by which E2 activates those circuits are not straightforward.

4.1. Experiment 1: rapid effects of E2 on aggression

In Experiment 1, E2 treatment significantly increased both attacking and time spent near the opponent in WS, but not TS birds (Fig. 3A, B). These results are consistent with our previous work showing that when plasma levels of T or E2 were experimentally equalized between the morphs, morph differences in behavior persisted. Maney et al. (2009) treated non-breeding male white-throated sparrows with subcutaneous silastic capsules containing T; females received capsules containing E2. Within seven days, the hormone stimulated singing in both sexes. Despite the fact that the capsules elevated plasma sex steroids to identical levels in the two morphs, WS birds of both sexes sang more than TS birds. In fact, E2-treated TS females did not sing at all; the treatment stimulated singing in WS females only. Thus, the morphs responded differently to the same dose of steroid, suggesting differential sensitivity. In that study, behavior was measured seven days after the onset of treatment, so we do not know how rapidly a morph difference in behavior could have been detected. Seven days of treatment allows ample time for steroid-induced changes in neural circuitry and many indirect effects. A study with a tighter time frame was needed.

In the current study, we found that E2 treatment affected aggression in WS birds within 20 min. This rapid effect was similar to that reported by Heimovics et al. (2015a) who found that in non-breeding song sparrows, the same oral dose of E2 rapidly increased attacking and time spent near the opponent. The time course of this effect is inconsistent with genomic

mechanisms of steroid action. Rather, it is consistent with nongenomic actions at cell membranes. Rapid, nongenomic actions of sex steroids are widely understood to be important for social perception and a variety of social behaviors (reviewed by Cornil et al., 2013; Ervin et al., 2015; Rudolph et al., 2016), in particular aggression (Heimovics et al., 2015b). Our current results suggest that the morph difference in aggression in this species may result, at least in part, from morph differences at the level of rapid E2 action. One such mechanism involves interactions between ERα and the metabotropic glutamate receptor mGlu1 (reviewed by Mermelstein, 2009); in white-throated sparrows, both of these genes are located inside the rearrangement of chromosome 2 and are differentially expressed in the brain (Zinzow-Kramer et al., 2015). How mGlu1 may contribute to behavioral morph differences will be the subject of future study.

The birds in this study were in non-breeding condition. Outside of the breeding season, birds of this species form hierarchies within flocks and regularly engage in aggressive behaviors such as singing and attacking. These behaviors do not depend on morph, however, during times of year when plasma sex steroids are low. As plasma levels of sex steroids increase in the spring, morph differences in these behaviors emerge (reviewed by Maney, 2008; Maney and Goodson, 2011). In this study, morph differences in behavior appeared in non-breeding birds after a bolus dose of E2, which the birds would not normally experience in the fall. This effect may be mediated by morph differences in the expression of sex steroid receptors. ERα mRNA is expressed at higher levels in WS than TS birds, in some brain regions, throughout the year (Horton et al., 2014b; Maney et al., 2015). The stability of morph differences in ERα expression is consistent with that reported for song sparrows, which showed a lack of seasonal changes in ERα and ERβ mRNA in the brain (Wacker et al., 2010). Results such as these have led to the hypothesis that seasonal changes in estrogen-dependent behaviors are likely mediated not by seasonal changes in ER abundance, but rather by local availability of E2. In the brains of song sparrows, for example, whereas ER levels remain the same, levels of aromatase increase dramatically in the spring (Soma et al., 2003; Wacker et al., 2010). Neural populations of ERα may therefore be stable and largely unoccupied in the fall, ready to respond to a large, bolus dose of E2 as was administered in this study.

The best-known behavioral morph difference in white-throated sparrows is in vocal aggression, particularly singing. During the breeding season, WS birds of both sexes sing more than TS birds (Horton et al., 2014a; Kopachena and Falls, 1993). In the present study, although it induced physical aggression, E2 treatment did not induce singing. Singing can, however, be induced in laboratory-housed non-breeding birds of this species by treatment with sex steroids for a longer duration, *e.g.* several days (Maney et al., 2009). This time course is similar to that reported for other songbirds. For example, laboratory-housed male canaries (*Serinus canaria*) require continuous exposure to elevated T for four days before they begin to sing, and reach singing rates typical of the breeding season after 11 days of T treatment (Sartor et al., 2005). Attempts to induce song by administering E2 rather than T have produced mixed results in a variety of species (Harding et al., 1988; Sartor et al., 2005; Soma et al., 2000; Tramontin et al., 2003) but in no case were rapid effects reported. Alward et al. (2016) showed that systemic inhibition of aromatase rapidly reduced song rates in canaries, suggesting that E2 is required for the moment-to-moment control of singing. In

that study, the birds had been photostimulated for one week and therefore were likely exposed to gonadal T during the days leading up to the behavioral tests. Together with our results, these studies suggest that rapid effects of E2 on vocal aggression, if they exist, may require priming by sex steroids. In that case, because WS birds sing at higher rates than TS birds, the behavioral polymorphism in white-throated sparrows cannot be completely explained by rapid effects of E2. The requirement for longer-term priming could be tested by administering a low dose of sex steroid for a period of days before administering a bolus dose of E2 (Cornil et al., 2006).

In this study, we tested the effects of E2 on aggression under two conditions: with and without visual contact with an opponent. In WS birds, E2 increased both attacking and time spent near the opponent during the period of visual contact. When a visual barrier was placed between the cages, the effect of E2 on attacking disappeared, but E2- treated WS birds continued to spend more time on the side of the cage near the opponent (Fig. 3B). This result is reminiscent of the phenomenon known as persistence, in which free-living birds subjected to a simulated territorial intrusion continue to exhibit vigilance even after the threat has been removed (Wingfield, 2005). In song sparrows, persistence can be observed for hours or even days after an intrusion and is thought to be mediated by sex steroids (Wingfield, 1994a, 1994b). Our behavioral paradigm does not exactly model the conditions under which persistence is usually studied, because the birds maintained auditory contact after the barrier was replaced. Our results are consistent with a role for E2 in the persistence of aggression after visual stimuli are withdrawn, however, and we saw evidence of such only in WS birds.

4.2. Experiment 2: rapid effects of E2 on Egr-1 expression

In Experiment 2, we tested whether the same bolus dose of E2 that affected behavior in Experiment 1 also altered Egr-1 expression in the brain. We focused in particular on the regions in which ERα expression depends on morph (Horton et al., 2014b). We found significant effects of E2 administration in only one region: TnA. In non-songbirds such as pigeons and quail, the region known as TnA is homologous to the mammalian medial amygdala (MeA; see Reiner et al., 2004). In songbirds, however, the region by the same name is entirely pallial and therefore not the MeA homolog (Vicario et al., 2017). Nonetheless, it shares many features with MeA. The songbird TnA is heavily inter-connected with regions that make up a social behavior network (Cheng et al., 1999; Goodson, 2005; Newman, 1999) and it expresses sex steroid receptors and aromatase (Bernard et al., 1999; Soma et al., 2003; Wacker et al., 2010). As a target of both the auditory pathway and the song system, it serves as an important hub for the integration of communication signals. In zebra finches, TnA lesions disrupt the ability to adjust singing behavior to social context (Ikebuchi et al., 2009), suggesting an important role in the perception of social cues.

The effect of E2 administration on Egr-1 expression in TnA was inhibitory (Fig. 4). This finding is consistent with that of Heimovics et al. (2012), who also reported inhibitory effects in TnA. They showed that in song sparrows, E2 rapidly decreased immunoreactivity for the phosphorylated form of ERK, a transcription factor that targets Egr-1 (Mayer and

Thiel, 2009; Shi et al., 2002). Both ERK and Egr-1 are part of the MAPK signaling cascade activated by membrane-associated ERs (reviewed by Kelly and Rønnekleiv, 2008; Sellers et al., 2015). Most research points to a stimulatory effect of E2 on this pathway, including in songbirds (Tremere et al., 2009). Like Heimovics et al. (2012), however, we found evidence of inhibitory effects. Attenuation of intracellular signaling by ER activation has been described, and the underlying mechanisms investigated *in vitro*. In mouse cortical cultures and nucleus-free preparations, specific ERα agonists have a profoundly inhibitory effect on phosphorylation of ERK (Singh et al., 2000; Toran-Allerand et al., 2002). *Via* a different pathway, both ERα and ERβ can interact with mGlu receptors to inhibit phosphorylation of CREB (Boulware et al., 2005; Mermelstein, 2009). The timing of measurement is important, because the activation of ERK phosphorylation by E2 can occur in an oscillatory fashion, with an initial increase followed by a decrease (Bulayeva et al., 2004). Because inhibiting the phosphorylation of ERK or CREB could ultimately attenuate Egr-1 expression, these results suggest multiple pathways by which E2 could attenuate the Egr-1 response. These effects are not, however, well-documented *in vivo*.

Because ERα expression varies according to morph in all of the brain regions we sampled in this study (Horton et al., 2014b), we expected that the Egr-1 response to E2 would likewise depend on morph. We did not, however, detect an interaction between morph and treatment even in TnA, a region with striking morph differences in ERα expression (Horton et al., 2014b; Maney et al., 2015). Because we sampled at only one time point and labeled only one marker, we may have missed an interaction that would have been detectable at a different time or using a different marker (see Bulayeva et al., 2004; Knight et al., 2012). It is important to note, however, that our manipulation did not target ERα specifically. TnA expresses high levels of GPER (formerly called GPR30) and ERβ (Acharya and Veney, 2012; Gahr, 2001; Gahr et al., 1993; Metzdorf et al., 1999). Neither of these genes are located inside the rearrangement of chromosome 2 in this species and their expression in TnA or hypothalamus does not depend on morph (Zinzow-Kramer et al., 2015). Like ERα, ERβ can inhibit CREB phosphorylation under the right conditions (reviewed by Mermelstein, 2009). Thus, if E2 increases aggression in WS birds by acting specifically on ERα circuits, we may not have detected an effect on Egr-1 expression despite the obvious effect on behavior. Stimulation of other ERs may have obscured changes in Erg-1 expression caused by ERα signaling alone. In future studies, ERα-specific agonists should be used in order to tease apart the contributions of ERα from those of other ERs.

Another possible explanation for the lack of an interaction between morph and hormone treatment is that we measured Egr-1 expression in birds that were not undergoing a social challenge. Because morph differences in behavior are observed during social encounters (*e.g.*, Horton et al., 2014a), social interaction might be necessary to see a morph difference in Erg-1 expression in the brain. In other words, E2 may modulate the neural response to social stimulation in a morph-specific way. We measured Egr-1 expression in the absence of social stimulation in order to map neural responses to E2 without introducing confounds associated with engaging in aggressive behavior, which differs by morph in birds in breeding condition (Horton et al., 2014a; Maney and Goodson, 2011). Thus, our experimental design intentionally ruled out possible effects of social interactions on Egr-1 expression. Morph-

typical neural responses to social stimulation may be required to observe morph differences in E2-induced Egr-1 expression.

5. Conclusions

In this study, E2 administration rapidly induced aggression in white-throated sparrows of the WS morph, but not the TS morph. This result adds to existing evidence suggesting that differential sensitivity to E2 plays a causal role in the behavioral polymorphism in this species. In a previous study (Maney et al., 2009), we showed that chronic E2 treatment increased aggressive behavior in WS but not TS birds. In the present study, effects were observed within 20 min after treatment, suggesting that nongenomic mechanisms of E2 action may contribute to morph differences in behavior.

The white-throated sparrow is an important model in behavioral neuroendocrinology because it is one of very few vertebrates in which genetic differentiation can be linked, through multiple levels of biological organization, to changes in behavioral phenotypes. For example, we previously showed that ESR1 promoters on the $ZAL2^m$ chromosome, which is found in WS but not TS birds, contain fixed polymorphisms that regulate transcription of ERα (Horton et al., 2014b). We showed further that the expression of ERα in TnA depends strongly on morph and fully predicts the effects of morph on aggression (Horton et al., 2014b). Behavioral studies in which E2 is manipulated, such as the current study, are critical to move beyond correlational evidence and show definitively that the effects of E2 on behavior also depend on morph. Manipulation of ERα expression will be necessary to tie the changes in ESR1 sequence directly to the evolution of behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2017.11.014.

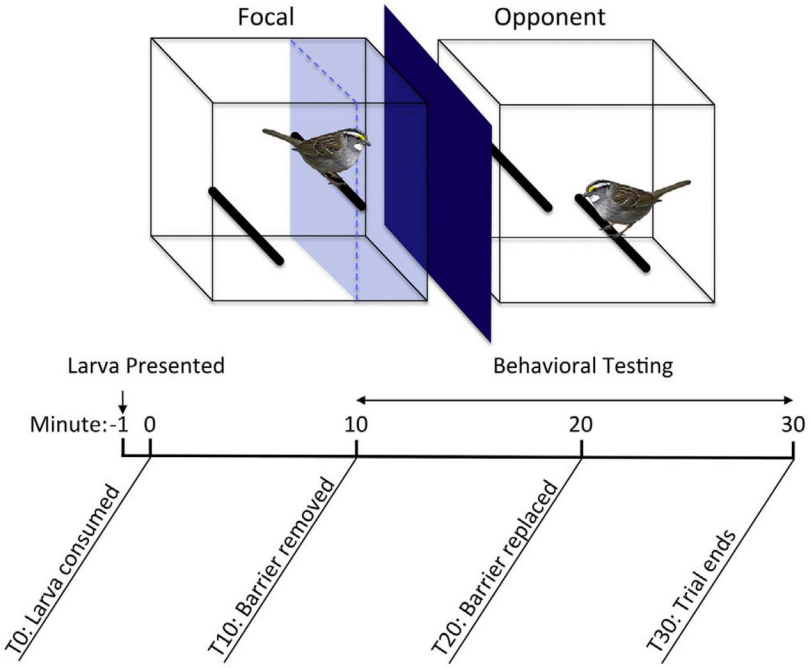

Fig. 1.

Paradigm for behavioral testing. The focal bird and opponent's cages were separated by a visual barrier (dark blue). The focal bird was presented with a larva injected with E2 or CON immediately before the onset of the trial, which began upon larva consumption (T0). Ten min later (T10), the visual barrier was removed, and the birds could interact visually and vocally (T10–T20). At T20, the visual barrier was replaced and the trial ended 10 min later (T30). One of the measures of aggression was time spent near the opponent, defined as the third of the cage closest to the opponent (light blue). The perch closest to the opponent marked the boundary of this area and was considered to be inside it (cage dimensions and bird not drawn to scale). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

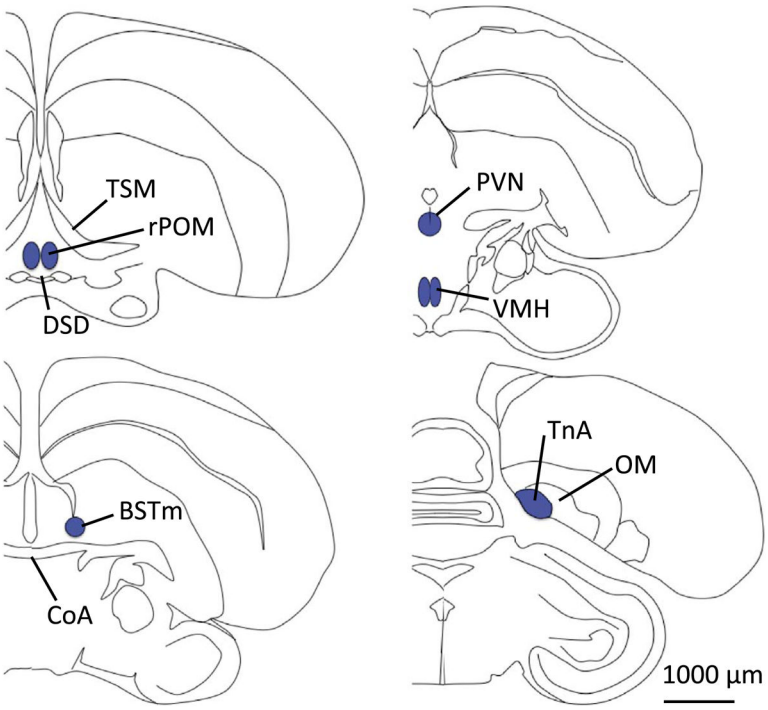

Fig. 2.

Regions of interest in Experiment 2. Egr-1 was quantified in the shaded regions. BSTm, medial portion of the bed nucleus of the stria terminalis; CoA, anterior commissure; DSD, supraoptic decussation; TnA, nucleus taeniae of the amygdala; OM, occipito-mesencephalic tract; PVN, paraventricular nucleus; rPOM, rostral medial preoptic area; TSM, septo-mesencephalic tract; VMH, ventromedial hypothalamus.

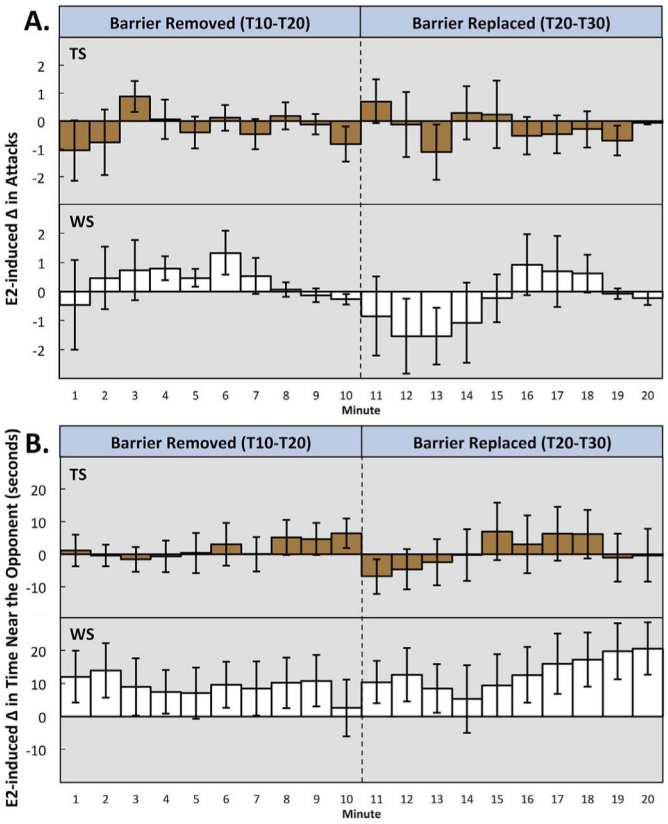

Fig. 3.

Rapid effects of E2 treatment on behavior. In order to visualize the results in terms of the main hypothesis that E2 affects the morphs differently, all scores were normalized by subtracting the baseline values (behavior during the CON trial) from experimental values (behavior during the E2 trial). The Y-axis thus depicts the *change* in behavior between the CON trial and the E2 trial. (A) E2 treatment increased attacks in WS birds ($p = 0.005$) but not TS birds ($p = 0.718$; morph × treatment interaction $p = 0.017$). After the barrier was replaced, this effect on attacking disappeared (morph × treatment interaction $p = 0.498$). (B) E2 increased the time spent near the opponent in WS birds ($p < 0.001$), but not in TS birds ($p = 0.804$; interaction $p = 0.022$). When the barrier was replaced, the effect of E2 persisted in WS birds ($p < 0.001$), but not TS birds ($p = 0.804$; morph × treatment interaction $p = 0.003$). Means and standard errors are plotted.

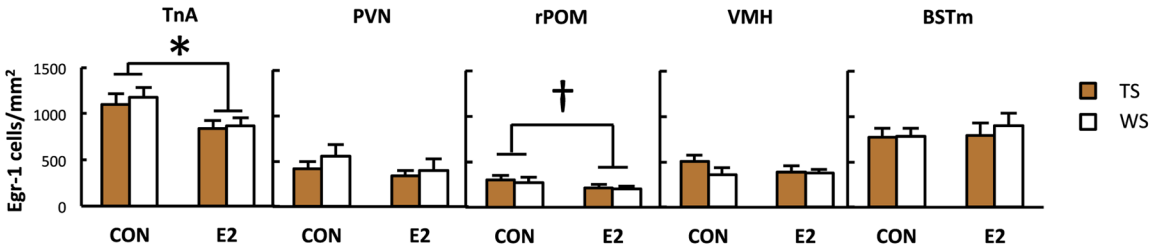

Fig. 4.

Effects of E2 treatment on Egr-1 expression in the brain. Egr-1-IR cells were counted in TnA, PVN, rPOM, VMH, and BSTm. A main effect of E2 treatment was found in TnA (* $p = 0.013$) and a trend was observed in rPOM († $p = 0.059$). No other effects were noted. For abbreviations, see the caption of Fig. 2. Means and standard errors are plotted.

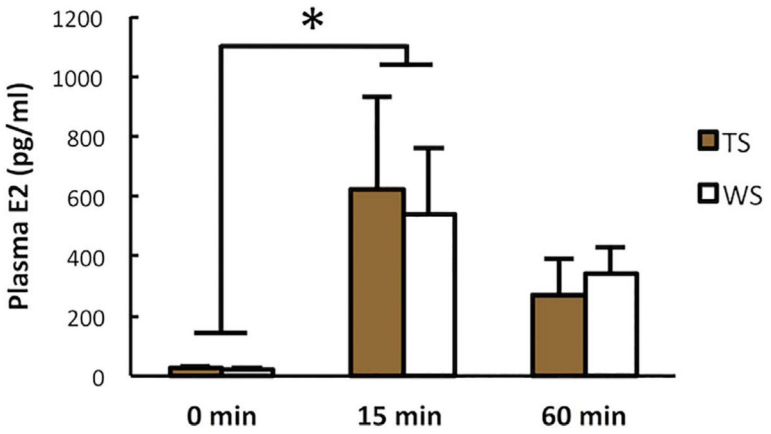

Fig. 5.

Plasma E2 levels after oral administration of E2. Oral administration of 300 μg E2 elevated plasma E2 significantly by 15 min ($p = 0.008$). Plasma E2 at 60 min tended to be lower than at 15 min, but this effect was not significant ($p = 0.161$). Oral administration affected plasma E2 to a similar extent in each morph; there was no interaction between morph and treatment ($p = 0.769$), or between morph, treatment, and time ($p = 0.834$). There was no main effect of morph ($p = 0.670$). Data at the 0 min time point are pooled from all CON birds. Means and standard errors are plotted.