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Temporal and bidirectional influences of estradiol on voluntary wheel running in adult female and male rats

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ABSTRACT

The sex steroid hormone 17β-estradiol (estradiol) regulates animal behavior as both a non-rapid hormone signal and as a rapid-acting neuromodulator. By practical necessity, estradiol's divergent temporal actions on rodent behavior are typically studied singularly and in one sex. We hypothesized that estradiol simultaneously acts through both temporal mechanisms to sex-specifically modulate a single behavior; and furthermore, that estradiol action in one temporal domain may regulate action in another. To test this hypothesis, we utilized one of the most robust rat behaviors exhibiting sex differences and estradiol-responsiveness, voluntary wheel running. Adult female and male rats were gonadectomized and exposed to daily repeated estradiol benzoate (EB) injections. Estradiol-sensitive running behavior was continually assessed in both the rapid and non-rapid temporal domains. We found that in female rats, estradiol rapidly decreased voluntary wheel running, but only after prior daily EB injections, supporting the hypothesis that non-rapid estradiol action influences rapid estradiol actions. Males exhibited a similar but less robust response, demonstrating sex-responsiveness. This rapid estradiol-induced decrease in running contrasted to non-rapid estradiol action which overall increased running in both sexes, revealing a bidirectional nature of estradiol's temporal influence. Non-rapid estradiol action also demonstrated sex-responsiveness, as a higher dose of EB was required to induce increased running in males compared to females. These findings indicate that estradiol rapidly, non-rapidly, and bidirectionally modulates wheel running in a sex-responsive manner, and that rapid estradiol action is modulated by non-rapid estradiol action. Overall, these data illustrate estradiol as a pleiotropic sex-responsive neuromodulator of a single behavior across temporal domains.

1. Introduction

Estrogens like 17β-estradiol (estradiol) exhibit complex signaling in the central nervous system. Estradiol is well-known for its role as a long-term hormonal signal, where it binds to nuclear estrogen receptors (ERs) α and β and induces profound changes in gene expression that changes neuron phenotype over a relatively slow timescale (hours to days, (Charlier et al., 2010; Mangelsdorf et al., 1995; O'Malley and Means, 1974)). Estradiol also induces rapid, neuromodulatory actions on neurons, where it acts through membrane estrogen receptors such as membrane-associated ERα and β and GPER-1 to facilitate changes in a neuron's functions through membrane protein interactions and second messenger systems (Meitzen and Mermelstein, 2011; Micevych et al., 2017; Srivastava and Evans, 2013). Rapid estradiol signaling of neurons is present in both sexes across vertebrates (Azcoitia et al., 2011; Callard et al., 1978; Kelly et al., 1976; Lord et al., 2009; Remage-Healey et al., 2012; Tuscher et al., 2016; Woolley, 2007) and can exhibit sex-specific receptor mechanisms (Krentzel et al., 2018; Oberlander and Woolley, 2016).

By practical necessity, the rapid and non-rapid effects of estradiol are typically studied independently and in one sex, especially given the divergent sex differences in estradiol exposure across the lifespan (McCarthy and Arnold, 2011). Importantly, estradiol could simultaneously act as a non-rapid and rapid modulator of a single behavior or neural function and modulate its own actions in either temporal domain. In considering this concept, we were drawn to the literature regarding the effects of daily estradiol injection (also called estrogen priming), wherein previous exposure to estradiol impacts future

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responsiveness to estradiol. For example, at the molecular level, repeated estradiol exposure impacts estradiol signaling via regulating ER expression levels and patterns (Catanuto et al., 2009; Hatae et al., 2009; Patisaul et al., 1999; Saceda et al., 1988; Sharma and Thakur, 2006). Regarding rapid estrogen action at the molecular level, exposure to masculinizing doses of estradiol during the organizational critical period eliminates later rapid estradiol action in female hippocampal neurons as assessed via CREB phosphorylation (Meitzen et al., 2012). These molecular findings are echoed at the behavioral level, where behaviors such as lordosis are maximally induced via multiple exposures to estradiol followed by progesterone (Micevych and Meisel, 2017; Micevych and Sinchak, 2018). More specifically, multi-day estradiol priming paradigms have also been shown to affect estradiol responsiveness to multiple behaviors such as female sexual receptivity and anxiety via membrane ERα and/or mGluRs (Christensen and Micevych, 2013; Dewing et al., 2007; Micevych and Dewing, 2011; Miller et al., 2020) and GPER-1 (Long et al., 2014). Another behavioral example comes from a study of the effects of prior daily estradiol injections on amphetamine-induced stereotyped motor behaviors and dopamine release in the striatum. This study finds that females previously exposed to estradiol for 3 days exhibited potentiated amphetamine-induced movements and dopamine release rapidly after a final estradiol injection compared to females who had a single estradiol injection or oil controls (Becker and Rudick, 1999). Overall, these findings suggested to us that sex-specific simultaneous rapid and non-rapid modulation of a single behavior by estradiol would most likely be revealed by employing a daily estradiol injection paradigm, with prior exposure to estradiol potentially inducing changes in a single behavior's responsiveness to rapid estradiol action. Broadly, we hypothesized that behavioral responsiveness to rapid estradiol signaling is dependent upon prior non-rapid estradiol exposure and exhibits sex-responsive effects.

To test our overall hypothesis, we employed voluntary wheel running in rats, which is a robust and consistent behavioral sex difference observed in behavioral neuroendocrinology. Observations of female rodents running more than males in wheels date to the early 1900s (Slonaker, 1912). These observations included that running wheel activity changes with the estrous cycle (Slonaker, 1924; Wang, 1923). This elevated running is abolished by ovariectomy and increased by repeated estradiol replacement both peripherally (Bowen et al., 2012; Rodier, 1971; Stern and Murphy, 1972) and in the brain (King, 1979; Spiteri et al., 2012). Male wheel running also can increase by ovary implantation (Wang et al., 1925) and estradiol replacement (Roy and Wade, 1975). To date no study has examined rapid-actions of estradiol on this behavior, despite the fact that several of the brain regions that control this behavior in adult rodents (Rhodes et al., 2003) express membrane estrogen receptors, including the striatal brain regions (Almey et al., 2012; Almey et al., 2015, 2016). Likewise, no study has attempted to assess rapid and non-rapid estradiol action on this behavior in the same animal. Using gonadectomy combined with repeated daily estradiol injections and continual behavioral monitoring, we tested whether estradiol rapidly and non-rapidly modulated voluntary wheel running across female and male rats and if temporal action in one domain is sensitive to that in another.

2. Methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University. Rats were housed in a temperature and light-controlled room (23 °C, 40% humidity, 12:12-hour reverse dark:light cycles) at the Biological Resource Facility of North Carolina State University. All cages are polysulfone Bisphenol A (BPA) free and filled with bedding manufactured from virgin hardwood chips (Beta chip, NEPCO, Warrengsburg, NY) to avoid endocrine disruptors present in corncob bedding (Mani et al., 2005; Markaverich et al., 2002; Villalon Landeros et al., 2012). Glass-bottle water and soy protein-free rodent chow (2020X, Teklad, Madison, WI USA) were provided *ad libitum*. Male and female Sprague Dawley CD IGS rats were born from timed-pregnant females purchased from Charles River Laboratories (Raleigh, NC). Rats were housed with littermates and dam until weaning. After weaning (P20–21), rats were grouped housed in same-sex cages with littermates until the beginning of each study. Rats were assigned into five studies. Litter origin was not analyzed as an experimental variable. For each study rats from 2 to 3 litters from different dams were distributed across treatment groups. Males and females from each litter were equally distributed across treatment groups. Some rats received injections of estradiol benzoate (EB; Sigma, St. Louis (Becker and Rudick, 1999)) or vehicle solution (oil). EB was subcutaneously administered at either 5 μg/0.1 mL (Becker and Rudick, 1999) or 50 μg/0.1 mL (Matsumoto et al., 2018) in a vehicle solution of 95% sesame oil/5% dimethyl sulfoxide (DMSO). Vehicle solution (oil) contained 95% sesame oil/5% dimethyl sulfoxide (DMSO) following Cao et al. (2016).

The following techniques are specific for each study described in this manuscript:

Study 1: Gonad-intact adult male (n = 5) and female (n = 7; age P90) rats were given access to running wheels in their individual home cages and recorded for 3 full estrous cycles. Each morning during the end of rats' subjective light/inactive period, estrous cycle stage was assessed in females via a wet mount preparation previously described (Hubscher et al., 2005; Proano et al., 2018). Males were also removed from their cages at this same time and handled in a similar manner to account for potential handling stress. All females exhibited normal estrous cycling with a cycle length of 4 days.
Study 2: Female adult rats were gonadectomized (P108 ± 2) and singly housed in home cages for 3 weeks (Fig. 1B). Each rat received 3 days of either 5 μg or a dose 10× higher at 50 μg EB injections 1 h

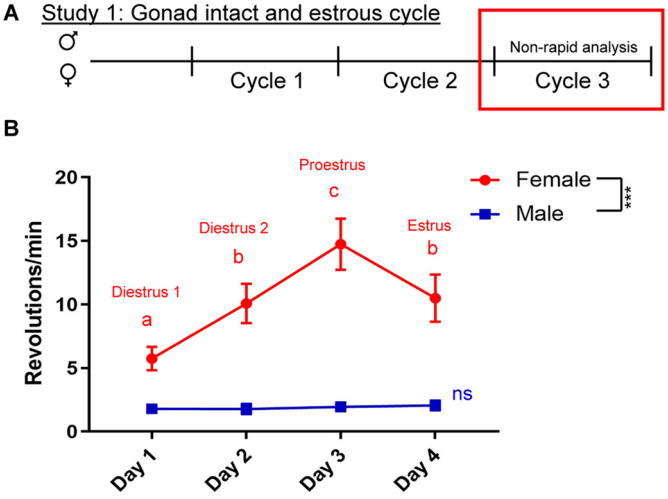

Fig. 1. Gonadally-intact females run more than males and voluntary wheel running is responsive to changes in the estrous cycle. A) Study 1 includes males (♂) and females (♀) with tracking female estrous stages across three cycles. The last cycle (red box) was analyzed for females and males that were day-matched. B) Females (red circles, n = 7) and males (blue squares, n = 5) average 12-hour dark phase running rate (revolutions/minute). Females had a higher running rate than males, and the rate changed with the estrous cycle phases with proestrus females having the highest running rate. *Statistics*: All data points presented as mean ± std. error. Letters represent within-subjects multiple comparisons per treatment group with different letters depicting significance. Asterisks represent between-subject comparisons between treatment groups $p < 0.0001^{***}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

after the start of the dark phase. These doses were designed to produce physiologically-relevant circulating estradiol levels at the time measured, because the low dose is similar to concentrations experienced during proestrus, and the high dose is similar to concentrations experienced during late pregnancy (Saddick, 2014; Smith et al., 1975). It is likely with both injection paradigms rats will experience elevated estradiol levels in the hours directly after injection beyond concentrations measured at sacrifice. These doses are also selected based on the strain of rat used, Sprague Dawley (Becker and Rudick, 1999), as estradiol dosing designed to mimic endogenous levels experienced during proestrus are strain dependent. 24 h later on Day 4, rats were euthanized and trunk blood was collected to measure circulating estradiol. This time point represents the same time point of rapid-estradiol analysis in Studies 3–5 and do not represent peak levels achieved. Serum from six proestrus females (P70–P80) naïve to injections were used as a positive control and 6 gonadectomized females (P70–P80) treated with oil injections were used as a negative control.

Study 3: Adult male (n = 12) and female (n = 12) rats were bilaterally gonadectomized on P55–57. Rats were briefly anesthetized with isoflurane and the testes or ovaries were removed. Rats recovered in single-housed cages with health monitoring for 3 weeks. Rats were then individually housed in cages containing a running wheel. All rats acclimated in wheel cages for one week before hormone injections. Rats were then divided into 4 groups: male vehicle (3 days of sesame oil injection + Day 4 50 μg EB injection, n = 6), female vehicle (3 days of sesame oil injection + Day 4 50 μg EB injection, n = 6), male EB (all 4 days 50 μg EB injection, n = 6), and female EB (all 4 days 50 μg EB injection, n = 6). Injections occurred 1 h after the start of the dark phase which is during the active running period for rats.

Study 4: Using the same females from Study 1 (n = 7, P130) after completing the estrous cycle recordings, rats were gonadectomized and then 3 weeks later moved to cages with running wheels. Rats were acclimated in wheel cages for one week before hormone injections. All rats received 3 days of 50 μg EB injections and on Day 4 an injection of oil vehicle. Injections occurred 1 h after the start of the dark phase.

Study 5: Male (n = 11) and female (n = 12) rats were treated in the same manner as Study 3, except 5 μg of EB was administered.

2.2. Running wheel data analysis

Wheels were constructed of stainless steel and were 34.3 cm in diameter. VitalView software (Starr Life Sciences Corp, Oakmont, PA) monitored wheel running activity and counted revolutions per minute across the entire period of the rat's access to the wheels. Wheel revolutions were assessed via a magnetic sensor. Wheel running was quantified in either 15-minute bins ("rapid analysis") or across the entire 12-hour active dark phase period ("non-rapid analysis"). For the rapid analysis, each subject's running was averaged in 15-minute bins to generate average revolutions/minute. Two 15-min bins were analyzed before injection to assess baseline activity. After injection, activity was analyzed in 15-minute bins for 2 h. For the non-rapid day analysis, the entire 12-hour active dark phase was summed and then averaged per min for each day's nocturnal period for an average revolutions/minute. For determining sex differences of responsiveness to daily estradiol injections, the 15–30 minute time bin after injections was selected for the rapid-phase and the 75–90 minute time bin after injections was selected for the non-rapid phase to subtract from the pre-injection average for each day.

2.3. Estradiol assay

Methods were adapted from a previously validated protocol (Chao et al., 2011; Tuscher et al., 2016). Briefly, at sacrifice, trunk blood was collected and immediately centrifuged at 2000 rpm for 20 min. Serum was then stored at −80 °C until extraction. 250 μL of serum was extracted twice using a 10:1 ratio of diethyl ether. Each sample was snap frozen with liquid nitrogen and the ether-containing organic compounds were poured off into clean glass tubes. All samples were dried overnight and resuspended in buffer from the enzyme-linked immunoassay kit (Cayman Chemical Estradiol ELISA Kit, Ann Arbor, MI). Samples were measured in duplicates including extraction efficiency controls using the Estradiol EIA kit. Extraction efficiency was 78% and intra-assay variability was 3.2%. All samples were above detectability for the assay.

2.4. Statistical analysis

Quantitative wheel running data were analyzed using a two-way mixed factor ANOVA (time within-subject and hormone treatment/sex as the between subject) and subsequent Tukey's HSD post-hoc tests (GraphPad Prism version 8, San Diego, CA) for Studies 1, 3, and 5. For study 2 we analyzed 17β-estradiol (E2) concentration using a one-way between subjects Brown-Forsythe ANOVA with subsequent one tailed *t*-tests with Welch's correction post-hoc tests to control for detected differences in standard deviation. For Study 4, we analyzed the oil-control experiment using a one-way within subject ANOVA. We further analyzed EB rats to assess sex-specific responsiveness by normalizing wheel running data in response to EB to the averaged pre-injection baseline and comparing via unpaired or one-sample *t*-tests, as appropriate. Significance is *a priori* considered at $p < 0.05$. Effect sizes are reported as partial eta-squared (η^2p) for F-tests and Cohen's d (d) for *t*-tests. Effect sizes are particularly useful for determining sex-responsive effects when both sexes have a similar directional effect but a difference in magnitude (Beltz et al., 2019). Mean (M) and mean difference (MD) are reported for descriptive statistics.

3. Results

3.1. Study 1: daily voluntary wheel running exhibited robust sex-specificity in gonad-intact rats

We calculated the average wheel running rate during the 12-hour dark phase of each day across different estrous cycle stages in females and matched days in males (Fig. 1A). Females across all stages of the estrous cycle ran much more than males (Fig. 1B; Sex: $F(1,10) = 23.3$, $p = 0.0007$, MD = 8.37 ± 1.73, $\eta^2p = 0.70$). As expected, daily voluntary wheel running rate changed across the female estrous cycle, with the highest average running rate observed in proestrus (Fig. 1B, Sex * Day: $F(3,30) = 10.7$, $p < 0.0001$, $\eta^2p = 0.52$; post hoc tests: Proestrus vs. all $p < 0.001$, Diestrus I vs. all $p < 0.001$, Diestrus II vs. Estrus $p = 0.97$). These findings serve as a key control experiment replicating previous laboratories' studies and provide an important foundation for our experiments employing controlled estradiol replacement.

3.2. Study 2: circulating estradiol levels induced via daily injections of 5 and 50 μg doses of EB

Circulating levels of serum estradiol was assessed for two doses of EB (5 μg/0.1 mL; 50 μg/0.1 mL) after 3 days of injections in gonadectomized females (Fig. 2A). Serum from gonadectomized females who received oil injections served as a negative control and serum from gonad-intact females in the proestrus phase of the estrous cycle served as a positive control. We first determined whether the proestrus females and the 5 μg and 50 μg dosed females were higher than gonadectomized oil controls. All three groups were higher than the oil controls, indicating that gonadectomy and sesame oil appropriately decreased circulating estradiol and both dosing paradigms increased circulating estradiol (Fig. 2B, Overall ANOVA: $W(3,9.29) = 10.9$, $p = 0.002$,

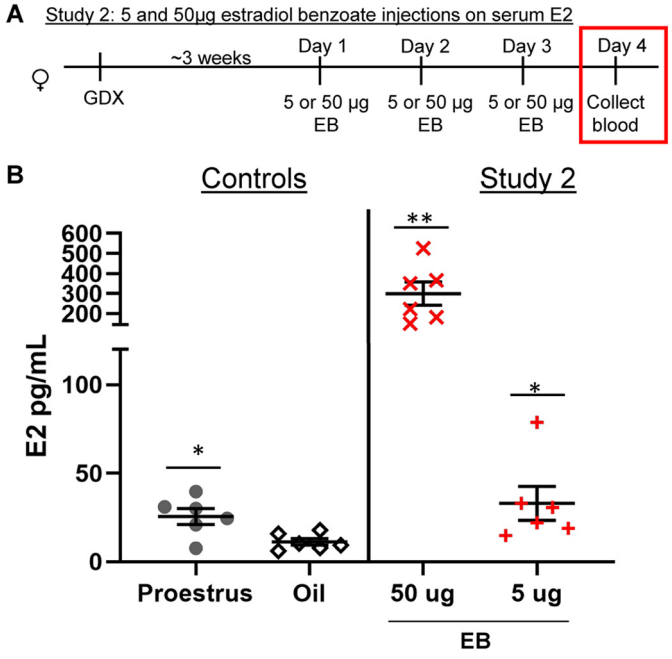

Fig. 2. 50 µg and 5 µg daily injections of EB on circulating estradiol. A) Study 2 includes females (♀) that were gonadectomized (GDX) and then three weeks later in their home cages given injections of either 5 µg or 50 µg of EB for 3 days (red box). B) Estradiol (E2 pg/mL) was measured from serum collected from females treated with 3 days of either 50 µg or 5 µg of estradiol benzoate (EB), serum from proestrus and gonadectomized females given oil was also used as positive and negative E2 controls respectively. 50 µg EB treated females had 10× more E2 than 5 µg EB and Proestrus females. *Statistics*: All data points presented as mean ± std. error. Asterisks indicate significance of oil group compared to all other groups $p < 0.05$*, $p < 0.01$**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$\eta^2p = 0.78$; post hoc tests: Oil vs. Proestrus $p = 0.011$, Oil vs. 50 µg $p = 0.002$, Oil vs. 5 µg $p = 0.036$). As expected, daily exposure to a 5 µg dose of EB in gonadectomized females induced circulating plasma levels of 17β-estradiol (E2, M = 33.0 ± 9.56 pg/mL) comparable to that of proestrus phase gonad-intact females (M = 25.6 ± 4.45 pg/mL). Females exposed to daily injections of 50 µg EB exhibited circulating E2 serum levels (M = 299.4 ± 57.8 pg/mL) that were approximately 10-fold higher than those exposed to the 5 µg dose. These findings indicate that this estradiol replacement paradigm is sufficient for studies analyzing estradiol's actions on voluntary wheel running.

3.3. Study 3: daily injection of 50 µg of EB induces non-rapid increases in voluntary wheel running and induces rapid sex-responsive decreases in voluntary wheel running

3.3.1. Non-rapid analysis

Rats receiving the 50 µg dose of EB exhibited a significant sex/treatment by day interaction (Sex/Trt ∗ Day: $F(9,60) = 6.17$, $p < 0.0001$, $\eta^2p = 0.48$). Oil treated gonadectomized males and females were similar in their low levels of voluntary wheel running (Fig. 3B, post hoc tests: Day 1 Oil Male vs. Oil Female $p = 0.65$) demonstrating the necessity of ovarian hormones for sex differences in voluntary wheel running. Rats exposed to 50 µg EB sequentially increased wheel running in both sexes (Fig. 3B, post hoc tests: EB Female Day 1 vs. Day 4 $p = 0.001$ and EB Male Day 1 vs. Day 4 $p < 0.0001$). Surprisingly, males and females exhibited similar magnitudes of elevated voluntary wheel running, including on the final injection day (Fig. 3B, post hoc tests: Day 4 EB Female vs. EB Male $p = 0.66$).

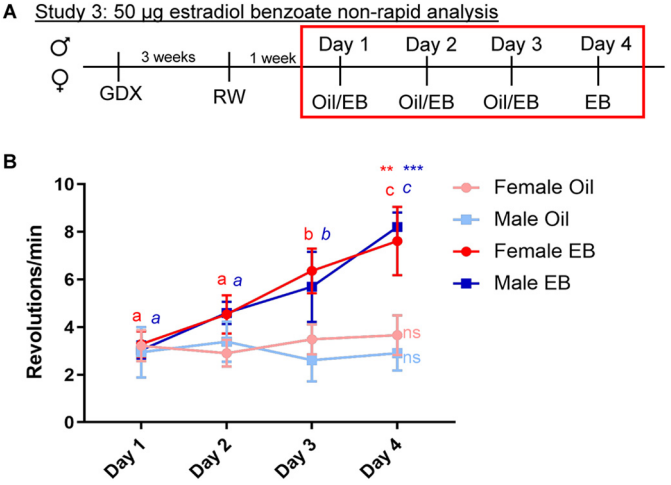

Fig. 3. 50 µg of EB non-rapidly increased voluntary wheel running across multiple days in both sexes. A) Study 3 includes males (♂) and females (♀) that were gonadectomized (GDX) 3 weeks before access to running wheel (RW). After one week of access, all rats received injections of either oil or 50 µg EB for three days and on day 4 every rats received EB. Non-rapid analysis is shown for all days (red box). B) Rats were separated into 4 treatment groups, female oil (light red circles, n = 6), male oil (light blue squares, n = 6), female EB (red circles, n = 6), and male EB (blue squares, n = 6) and displayed is the average 12-hour dark phase running rate (revolutions/minute) during injection days where 50 µg of EB was administered. Both female and male EB rats increased average daily running rate with each day of EB administration. *Statistics*: All data points presented as mean ± std. error. Letters represent within-subjects multiple comparisons per sex/treatment group with different letters depicting significance. Asterisks represent between-subject comparisons between treatment groups. Red asterisks and blue asterisks are Female EB vs. Female Oil Day 4 and Male EB vs. Male Oil Day 4 respectively. $p < 0.01$**, $p < 0.001$***. ns = not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2. Rapid analysis

We next analyzed whether estradiol induced a rapid, sex-specific effect on voluntary wheel running in females and males. Rats were analyzed on Day 1 and Day 4 of injection (Fig. 4A). For gonadectomized males and females exposed to 50 µg of EB or oil, on Day 1 we detected a significant main effect of time (Fig. 4B&C, Time: $F(9,180) = 3.56$, $p = 0.0004$, $\eta^2p = 0.15$, post hoc tests: all comparisons $p > 0.05$) and no sex/treatment by time interaction (Fig. 4B&C, Sex/Trt ∗ time: $F(27,180) = 1.12$, $p = 0.36$, $\eta^2p = 0.14$). This effect was largely driven by a small increase in wheel running occurring in the first 15-minute bin after injection on Day 1. This small increase in running was consistent across injection type and sex, indicating a possible stress response independent of EB. This short burst of running post-injection across treatment groups indicated that EB did not change voluntary wheel running rapidly on Day 1.

In contrast, on Day 4, we detected a significant sex/treatment by time interaction (Sex/Trt ∗ Time: $F(27,180) = 1.68$, $p = 0.025$, $\eta^2p = 0.20$), where females who received prior daily EB injections exhibited a significant decrease in voluntary wheel running beginning within the first 15 min of estradiol exposure and lasting approximately 1 h after EB injection (Fig. 4D, Females only Treatment ∗ Time: $F(9,90) = 3.024$, $p = 0.003$, $\eta^2p = 0.23$; post hoc tests: Female EB P 0–15 min vs. I 15–30 min $p = 0.003$, Female EB P 0–15 min vs. I 30–45 min $p = 0.018$, Female EB P 15–30 min vs. I 15–30 min $p = 0.026$). Females receiving daily injections of oil had no significant response to EB (Fig. 5D, post hoc tests: all time comparisons $p > 0.05$). On Day 4, compared to oil controls, females who received prior daily EB injections had significantly higher voluntary wheel running rates than oil controls (Fig. 4D, post hoc tests: Female EB P 15–30 min vs. Female Oil P 15–30 min $p = 0.04$). For males who received prior daily EB

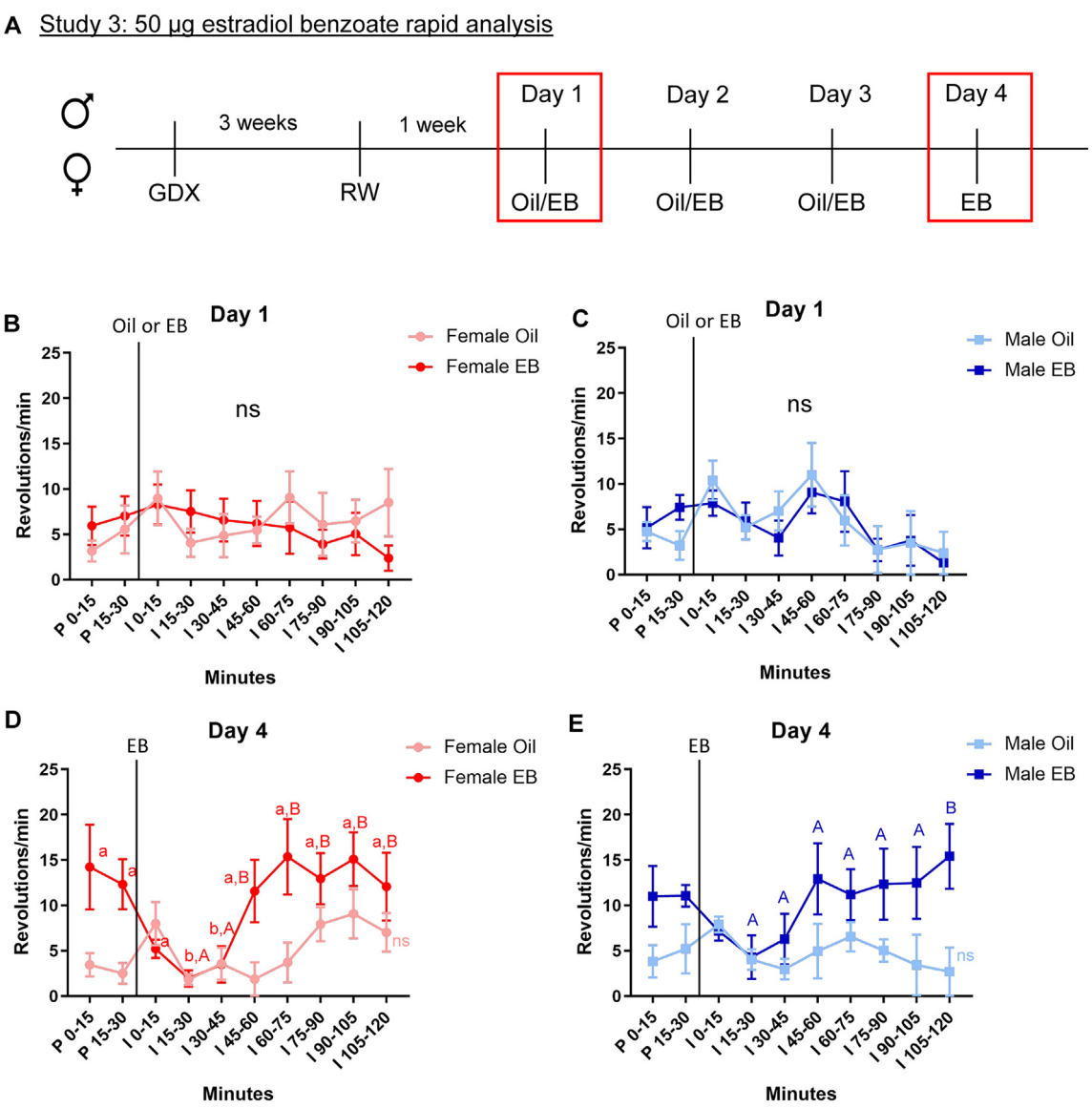

Fig. 4. Females and males that received prior daily EB injection at 50 µg rapidly decreased voluntary wheel running to EB. A) Study 3: Details are the same as previous figure except that rapid analysis is depicted for Days 1 and 4 (red boxes). B) Day 1 females injected with oil (light red circles, n = 6) or 50 µg EB (red circles, n = 6) 1 h after lights off. Running rate is depicted as the average revolutions/minute in 15-minute bins. No significant changes to running rate occurred. C) Day 1 males injected with oil (light blue squares, n = 6) or 50 µg EB (blue squares, n = 6) also did not have significant changes after injections. D) Day 4 females previously exposed to 3 days of oil (light red circles, n = 6) or 3 days of 50 µg EB (red circles, n = 6) were injected with 50 µg of EB 1 h after lights out. Females with prior daily estradiol injected EB had a significant decrease in voluntary wheel running 15–45 min after EB injection where oil females did not have significant changes after EB injection. E) Day 4 males previously exposed to 3 days of oil (light blue squares, n = 6) and 3 days of 50 µg EB (blue squares, n = 6) were injected with 50 µg of EB 1 h after lights out. Males with prior daily estradiol injected had a significant decrease in voluntary wheel running when injected with EB where oil males did not have significant changes after EB injection. *Statistics*: All data points presented as mean ± std. error. Letters represent within-subjects multiple comparisons per treatment group with different letters depicting significance. ns = not significant. P = pre-injection time period. I = after injection time period. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

injections, there was also a decrease in voluntary wheel running, though not as robust (Fig. 4E, Males only Treatment * Time: F (9,90) = 1.45, p = 0.18, η^2p = 0.13, post hoc tests: Male E I 15–30 min vs. I 105–120 min p = 0.042). These findings indicated that estradiol rapidly decreased voluntary wheel running dependent on non-rapid prior daily EB injections. This rapid action is sex-responsive, with females exhibiting a more robust decrease in running compared to males.

3.3.3. Sex-responsiveness across each day for rapid and non-rapid timepoints

We calculated the voluntary wheel running rate difference 15–30 min after injection for assessing rapid estradiol sex sensitivities and 75–90 min after injection for assessing non-rapid estradiol sex sensitivities. Both differences were normalized to the pre-injection average running rate for each day for males and females that received EB injections every day. Females exhibited significant rapid decreases in running on Days 2–4 (Fig. 5B, Day 1 t(5) = 0.99, p = 0.37, d = 0.41; Day 2 t(5) = 2.92, p = 0.033, d = 1.19; Day 3 t(5) = 3.39, p = 0.019, d = 1.38; Day 4 t(5) = 3.49, p = 0.017, d = 1.43). Males did not have a detectable rapid decrease until Day 4 (Fig. 5B, Day 1 t(5) = 0.33, p = 0.75, d = 0.14; Day 2 t(5) = 0.43, p = 0.68, d = 0.18; Day 3 t (5) = 1.12, p = 0.31, d = 0.46; Day 4 t(5) = 3.23, p = 0.023, d = 1.32). There were no significant differences in non-rapid actions detected for either males or females (Fig. 5C, Days 1–4 p > 0.05 for both sexes) which represents both sexes returning to the elevated baseline running rate by this time point.

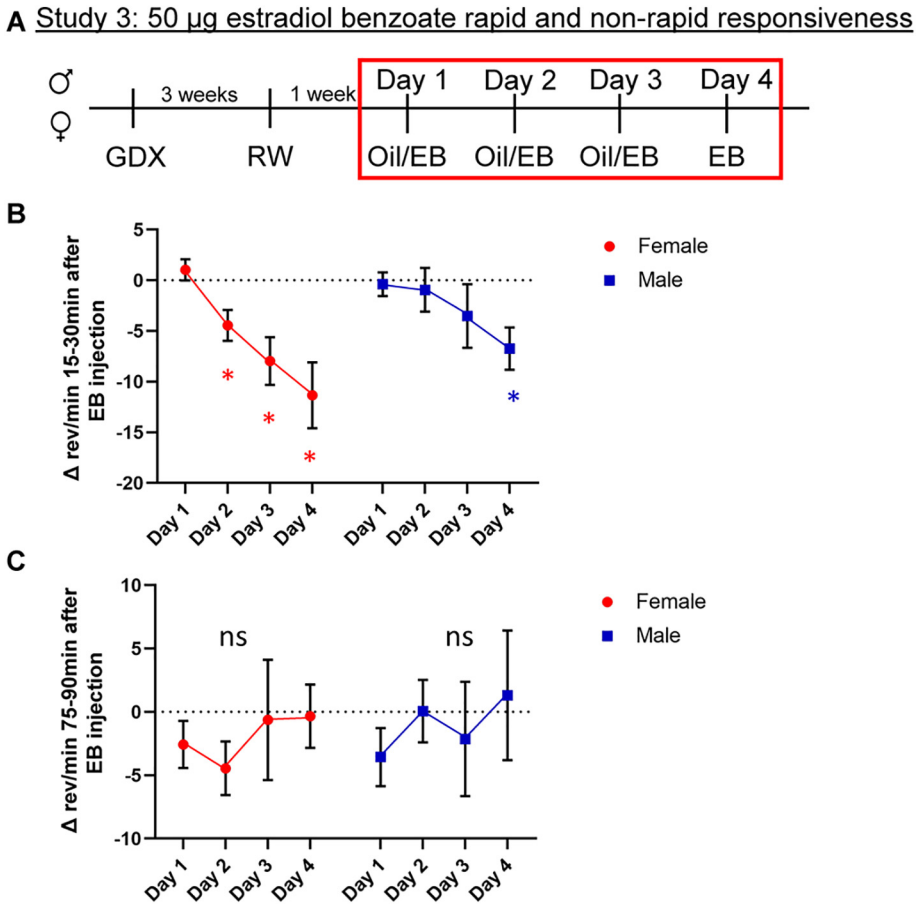

Fig. 5. Females are more responsive to the rapid actions of 50 µg of EB than males. A) Study 3: details are the same as Fig. 3. B) Female 50 µg EB (red circles, n = 6) and male 50 µg EB (blue squares, n = 6) running wheel rates at 15–30 min after injections were subtracted from the pre-injection average for each day of injections. Females had significant decreases in running wheel rate Days 2–4 while males only decreased Day 4. C) Female 50 µg EB (red circles, n = 6) and male 50 µg EB (blue squares, n = 6) running wheel rates at 75–90 min after injections were subtracted from the pre-injection average for each day of injections. Neither sex had significant changes, indicating that running rates had returned to pre-injection baseline. *Statistics*: All data points presented as mean ± std. error. Asterisks indicate significance. Red asterisks represent female day comparisons to 0 while blue represents male day comparisons to 0. $p < 0.05$*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Study 4: a daily dose of 50 µg EB does not induce a rapid voluntary wheel running response to oil injection

One alternative explanation for the rapid decrease in voluntary wheel running in response to the 50 µg dose of EB is due to a change in stress response generated by the prior daily EB injections. To control for this possibility, we exposed females to daily injections of 50 µg of EB for 3 days and on Day 4 injected oil (Fig. 6A). In contrast to our previous findings of EB rapidly decreasing voluntary wheel running after prior daily EB injections, rats injected with three daily injections of EB did not exhibit a change in wheel running in response to a fourth injection of oil (Fig. 6B, $F(3.57, 21.4) = 2.02$, $p = 0.13$, $\eta^2p = 0.14$). This critical control experiment indicates that the rapid decrease in voluntary wheel running activity in response to 50 µg of EB is specific to EB, and not due to an overall difference in stress response generated by the prior daily 50 µg EB injections.

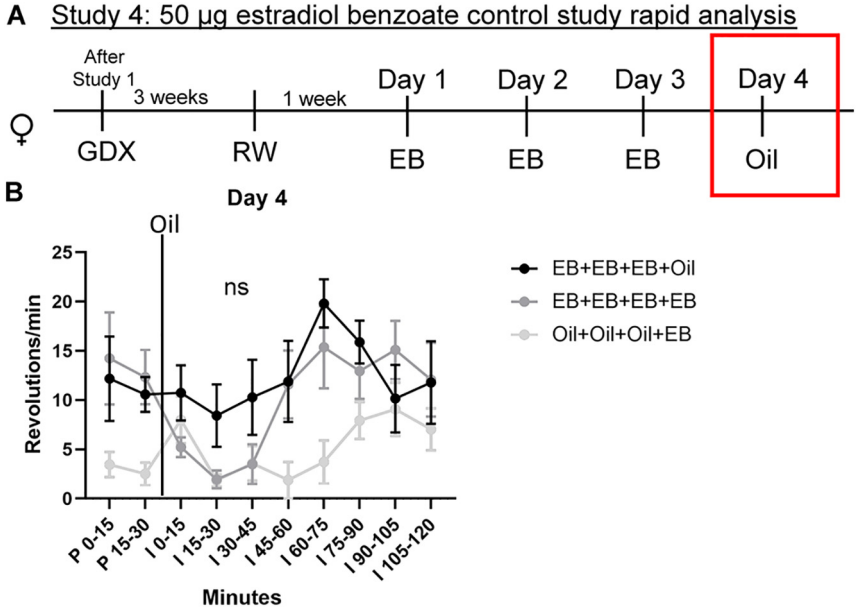

Fig. 6. Females that received prior daily EB injections at 50 µg did not rapidly decrease running to an oil injection. A) Study 4 follows the same design as Study 3, except only one group of females (♀) are given 3 days of 50 µg EB and on Day 4 they receive oil (red box) to test is there is a stress response after receiving 3 days of 50 µg EB. B) Females (black circles, n = 7) that received oil on Day 4 had no rapid significant changes to voluntary wheel running. Light gray lines in the background represent the same data from Fig. 4D only as a graphical comparison and not for statistical purposes. ns = nonsignificant. P = pre-injection time period. I = after injection time period. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

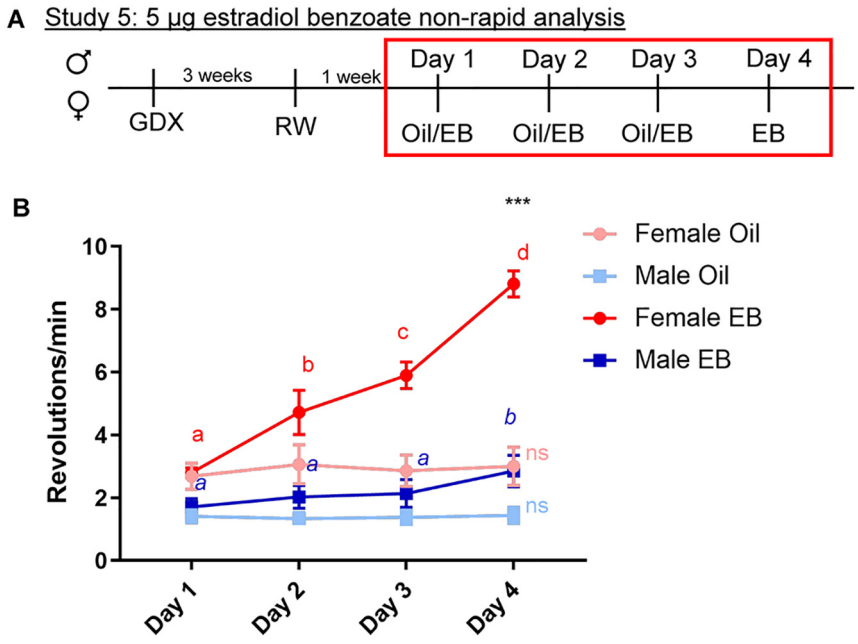

Fig. 7. 5 µg of EB non-rapidly increased voluntary wheel running across multiple days in females compared to males. A) Study 5 includes males (♂) and females (♀) that were gonadectomized (GDX) 3 weeks before access to running wheel (RW). After one week of access, all rats received injections of either oil or 5 µg EB for three days and on day 4 every rats received EB. Non-rapid analysis is shown for all days (red box). B) Rats were separated into 4 treatment groups, female oil (light red circles, n = 6), male oil (light blue squares, n = 5), female EB (red circles, n = 6), and male EB (blue squares, n = 6) and displayed is the average 12-hour dark phase running rate (revolutions/minute) during injection days where 5 µg of EB was administered. Female EB rats increased average daily running rate with each day of EB administration, where male EB rats did not until Day 4 and rate remained lower than female EB rats. *Statistics*: All data points presented as mean ± std. error. Letters represent within-subjects multiple comparisons per treatment group with different letters depicting significance. Asterisks represent between-subject comparisons between treatment groups. Red asterisks and blue asterisks are Female EB vs. Female Oil Day 4 and Male EB vs. Male Oil Day 4 respectively. Black asterisks represent Female EB vs. Male EB. $p < 0.001$***. ns = not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Study 5: daily injections of 5 µg of EB induces non-rapid sex-responsive increases in voluntary wheel running and does not induce a rapid change in voluntary wheel running

3.5.1. Non-rapid analysis

Rats receiving the lower 5 µg dose of EB exhibited a significant and robust sex/treatment by day interaction (Sex/Treatment * Day: $F(9,57) = 24.98$, $p < 0.0001$, $\eta^2p = 0.80$). Oil treated gonadectomized males and females were similar in their low levels of voluntary wheel running (Fig. 7B, post hoc tests: Day 1 Oil Male vs. Oil Female $p = 0.19$). Interestingly, rats exposed to the 5 µg EB dose exhibited strong sex-specific effects. For females, each day of EB injection sequentially elevated running, similar to the effects of the 50 µg dose of EB (Fig. 7B, post hoc tests: all day comparisons $p < 0.01$, EB Day 1 vs. EB Day 4 $p < 0.0001$). For males, each day of EB injection did not sequentially elevate running, with no detectable increase until Day 4 (Fig. 7B, post hoc tests: E Day 1 vs. E Day 4 $p = 0.005$). For Day 4, EB-treated females ran significantly more than males (Fig. 7B post hoc tests: Day 4 Female EB vs. Male EB $p < 0.0001$). Overall, these experiments indicate 5 µg of EB induced a non-rapid increase in voluntary wheel running in a sex-responsive manner.

3.5.2. Rapid analysis

We next analyzed whether the 5 µg dose of EB induced a rapid change in voluntary wheel running on Day 1 and Day 4 of injection (Fig. 8A). For gonadectomized males and females exposed to 5 µg of EB or oil, on Day 1 we detected a significant main effect of time (Fig. 8B&C, Time: $F(4.95,94.13) = 3.78$, $p = 0.0037$, $\eta^2p = 0.17$, post hoc tests: P 0–15 min vs. I 0–15 min $p = 0.009$) and no sex/treatment by time interaction (Fig. 8B&C, Sex/Trt * Time: $F(27, 171) = 1.05$, $p = 0.40$, $\eta^2p = 0.14$). Similar to experiments involving 50 µg of EB, rats exhibited a small increase in wheel running occurring in the first 15-minute bin after injection, indicating an effect independent of EB. For Day 4, we detected a significant sex/treatment by time interaction (Sex/Trt * Time: $F(27,171) = 1.98$, $p = 0.0048$, $\eta^2p = 0.24$), with EB females exhibiting larger difference in wheel running compared to EB males. However, EB females did not exhibit a sustained elevated response until 75 min after EB injection outside of the rapid time period (Fig. 8D, post hoc tests: Female EB P 0–15 min vs. I 75–90 min $p = 0.0059$, P 0–15 min vs. 90–105 min $p = 0.021$). Similar to Day 1, all females exhibited a small increase in wheel running occurring in the first 15-minute bin after injection, indicating an effect independent of estradiol. Males did not have any detectable changes in either oil controls or estradiol exposed (Fig. 8E, post hoc tests: all comparisons $p > 0.05$). These findings indicate that the dose of the daily injection of estradiol is instrumental for inducing responsiveness to rapid estradiol action in voluntary wheel running.

3.5.3. Sex-responsiveness across each day for rapid and non-rapid time-points

For rats exposed to daily injections of 5 µg of EB, there was no significant rapid difference in running detected for males (Fig. 9B, Days 1–4 $p > 0.05$). For females only, Day 2 exhibited a significant rapid increase in running (Fig. 9B, Day 2 $t(5) = 2.93$, $p = 0.033$, $d = 1.19$, post hoc tests: Day 1,3, and 4 $p > 0.05$), which was not maintained during the following days of exposure. For the non-rapid time point, females exhibited significant increases on Days 3 and 4 (Fig. 9C, Day 1 $t(5) = 0.90$, $p = 0.41$, $d = 0.37$; Day 2 $t(5) = 1.34$, $p = 0.24$, $d = 0.54$; Day 3 $t(5) = 2.76$, $p = 0.040$, $d = 1.13$; Day 4 $t(5) = 9.12$, $p = 0.0003$, $d = 3.72$). Males did not exhibit a detectable increase until Day 4 (Fig. 9C, Day 1 $t(5) = 1.95$, $p = 0.11$, $d = 0.80$; Day 2 $t(5) = 0.79$, $p = 0.47$, $d = 0.32$; Day 3 $t(5) = 1.08$, $p = 0.33$, $d = 0.44$; Day 4 $t(5) = 2.92$, $p = 0.033$, $d = 1.19$). On Day 4, females expressed a significantly higher increase in the voluntary wheel running rate than males (Fig. 9C, $t(10) = 5.02$, $p = 0.0005$, $d = 2.90$).

3.6. Analysis across studies 1, 3, and 5: higher levels of circulating estradiol in gonadectomized females are necessary to more accurately mimic the magnitude of voluntary wheel running exhibited by gonad-intact female rats

Notably, on Day 4, females that received daily 5 µg EB injections did not maintain an elevated voluntary wheel running rate during the baseline period before the injection (Fig. 8D), unlike what was observed with the 50 µg dose (Fig. 4D). We reasoned that the 5 µg dose may not accurately mimic the natural levels of voluntary wheel running displayed by a gonad-intact female, thus reducing the ability to detect a rapid effect of EB due to a floor effect. To test this possibility, we analyzed whether the Day 4 early dark-period/pre-injection levels of voluntary wheel running of females exposed to 50 µg (Study 3) or 5 µg EB (Study 5) were more representative of the levels of voluntary wheel running in gonad-intact proestrus females (Study 1). We compared average running rate across the 30 minute pre-injection for 50 µg or

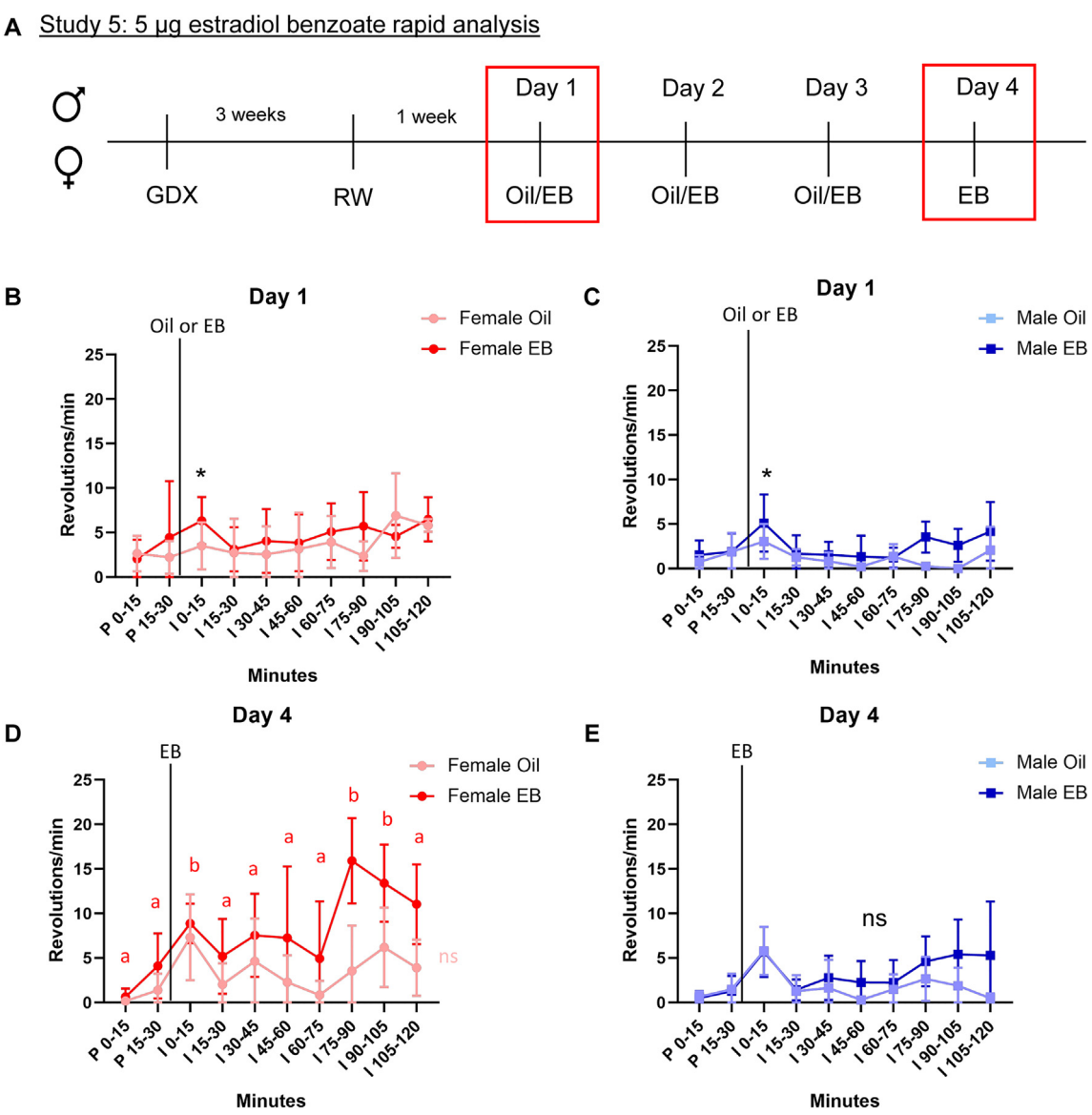

Fig. 8. Females that received prior daily EB injections at 5 µg did not rapidly change voluntary wheel running. A) Study 5: Details are the same as Fig. 7 except that rapid analysis is depicted for Days 1 and 4 (red boxes). B) Day 1 females injected with oil (light red circles, n = 6) or 5 µg EB (red circles, n = 6) 1 h after lights off. Running rate is depicted as the average revolutions/minute in 15-minute bins. No significant changes to running rate occurred. C) Day 1 males injected with oil (light blue squares, n = 5) or 5 µg EB (blue squares, n = 6) also did not have significant changes after injections. D) Day 4 females previously exposed to 3 days of oil (light red circles, n = 6) or 3 day of 5 µg EB (red circles, n = 6) were injected with 5 µg of EB 1 h after lights out. Females that received prior daily EB injections had a significant increase in voluntary wheel running 75–90 min after EB injection where oil females did not have significant changes after EB injection. E) Day 4 males previously exposed to 3 days of oil (light blue squares, n = 5) and 3 days of 5 µg EB (blue squares, n = 6) were injected with 5 µg of EB 1 h after lights out. Males who received prior daily EB injections and oil males did not have significant changes after EB injection. *Statistics*: All data points presented as mean ± std. error. Letters represent within-subjects multiple comparisons per treatment group with different letters depicting significance. ns = not significant. P = pre-injection time period. I = after injection time period. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5 µg EB exposed females from Study 3 and 5 and selected the same time period for intact proestrus females from Study 1. We found that proestrus females had higher running rates than females exposed to 5 µg EB (Fig. 10, $F(2,16) = 1.35$, $p = 0.0026$, $\eta^2p = 0.14$, post hoc tests: Proestrus vs. 5 µg EB $p = 0.0018$). Proestrus females expressed similar running rates to females exposed to 50 µg EB (Fig. 10, post hoc tests: Proestrus vs. 50 µg EB $p = 0.19$). This finding indicates that the higher dose of 50 µg EB daily injections is more appropriate at mimicking the voluntary wheel running rates observed during proestrus, despite the 5 µg dose being more representative of the physiological levels of circulating estradiol during proestrus (Fig. 2B).

4. Discussion

The findings presented here illustrate the pleiotropic action of estradiol across temporal domains on a single behavior. This broad conclusion is encompassed by three primary findings of estradiol actions on voluntary wheel running: 1) non-rapid and rapid actions of estradiol are both present and are bidirectional, 2) the presence of rapid actions of estradiol are contingent upon previous non-rapid actions, and 3) both non-rapid and rapid actions of estradiol are sex-responsive. Regarding non-rapid estradiol actions, estradiol increased voluntary wheel running with females exhibiting a more robust response to 5 µg of EB compared to males. Regarding rapid estradiol actions, estradiol acutely decreased voluntary wheel running but only in rats who received prior

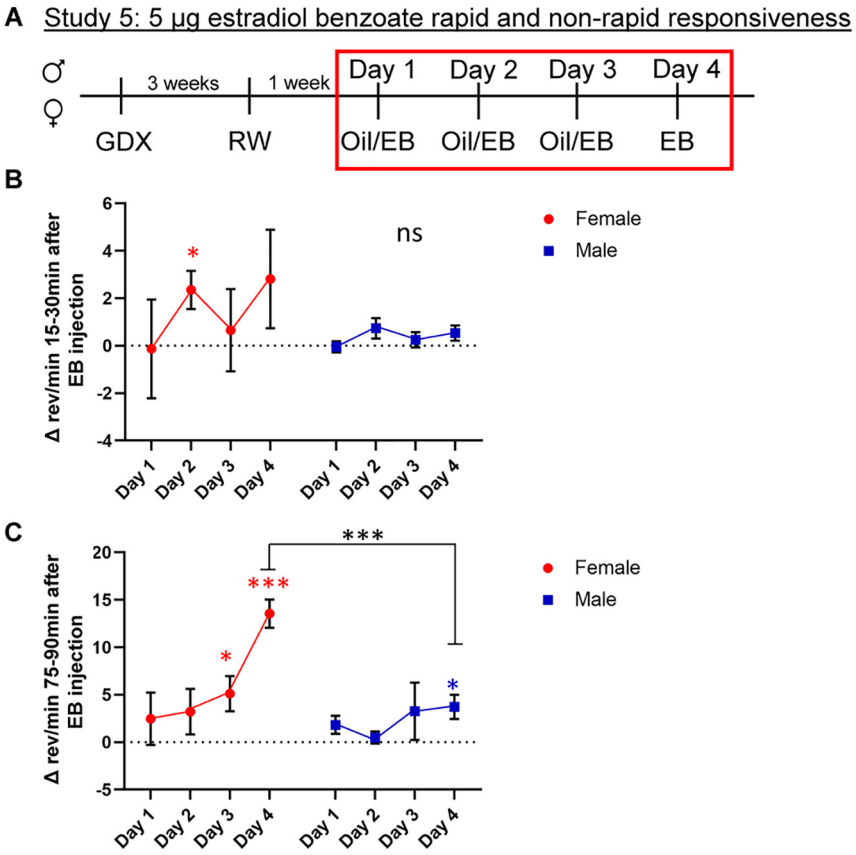

Fig. 9. Females are more responsive to the non-rapid actions of 5 µg of EB than males. A) Study 5: Details are the same as Fig. 7. B) Female 5 µg EB (red circles, n = 6) and male 5 µg EB (blue squares, n = 6) running wheel rates at 15–30 min after injections were subtracted from the pre-injection average for each day of injections. Females had significant increase on Day 2 in running wheel rate and males had no significant differences. C) Female 5 µg EB (red circles, n = 6) and male 5 µg EB (blue squares, n = 6) running wheel rates at 75–90 min after injections were subtracted from the pre-injection average for each day of injections. Females had significant increases in running wheel rate Days 3–4 while male only increased by Day 4. Day 4 female rates were higher than Day 4 male rates. *Statistics*: All data points presented as mean ± std. error. Asterisks indicate significance. Red asterisks represent female day comparisons to 0 while blue represents male day comparisons to 0. Black asterisks represent a sex comparison. $p < 0.05$*, $p < 0.001$***. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

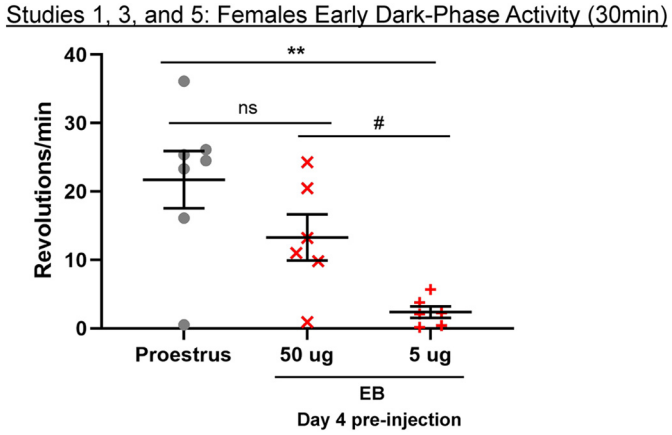

Fig. 10. Higher doses of EB replacement is required to replicate early-dark phase running rates of proestrus females. Groups pulled from multiple studies included proestrus females (Study 1, gray circles, n = 7), Day 4 Females 50 µg EB (Study 3, red x, n = 6), and Day 4 Females 5 µg EB (Study 5, red plus, n = 6) and the average running rate 30 min after lights out and before Day 4 injection was compared. Proestrus and Day 4 Females 50 µg EB had higher running rates than Day 4 Females 5 µg EB. *Statistics*: All data points presented as mean ± std. error. Asterisks represent between-subject comparisons between treatment groups. $0.05 > p < 0.10$#, $p < 0.001$**, ns = non-significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

daily 50 µg of EB. This finding indicated that the rapid actions of estradiol on voluntary wheel running were dependent on previous non-rapid actions of estradiol. Females also exhibited increased responsiveness compared to males to the rapid actions of estradiol, as females exhibited an acute decrease in running sooner than males.

To our knowledge, this is the first demonstration of a rapid modulation of voluntary wheel running by estradiol. Previous studies that tested estradiol replacement effects on voluntary wheel running tended to examine day to day or week to week averages of wheel running in rodents. They also consistently found that estradiol increased voluntary wheel running within a non-rapid timeframe (Bowen et al., 2012; Rodier, 1971; Spiteri et al., 2012; Stern and Murphy, 1972). We replicated these findings in our analyses that targeted non-rapid estradiol action in both sexes (Figs. 3B & 7B). This literature along with a previous study that demonstrated rapid-estradiol increased amphetamine-induced head and forelimb stereotyped motor movements (Becker and Rudick, 1999) indicated that estradiol typically *increases* locomotion both non-rapidly and rapidly. Instead, we found that estradiol's rapid actions acutely *decreased* voluntary wheel running, and this decrease was more robust in females who received prior daily estradiol injections. With each subsequent day of injection, female rats displayed a higher magnitude of the rapid decrease to estradiol. This rapid decrease in voluntary wheel running was specific to the acute effect of Day 4's exposure to estradiol, as females that received prior daily estradiol injections did not decrease running with an oil injection on Day 4 (Fig. 6B). This critical control experiment demonstrated that the rapid decrease is induced by estradiol and is not a non-specific response to a fourth injection regardless of content.

Interestingly, the rapid decrease in voluntary wheel running was only observed after prior daily 50 µg estradiol injections. This augmented responsiveness to rapid estradiol signaling may be due to two non-mutually exclusive hypotheses: 1) rapid estradiol responsiveness increases along with circulating estradiol levels or 2) rats require a high running rate in order to observe a decrease in voluntary wheel running. Regarding the first hypothesis, there is sufficient evidence generated by this study to conclude that previous exposure to estradiol increases the magnitude of running responsiveness to estradiol. For females, whether considering estradiol's rapid response (Fig. 5B) or estradiol's non-rapid response (Fig. 9C), each day of injection led to a change in voluntary

wheel running becoming more pronounced. Estradiol benzoate is metabolized slower than 17β-estradiol and can take 48–72 h to completely clear the body (Curtis, 2015; Oriowo et al., 1980), so repeated daily injections of EB would likely induce a stepwise increase of circulating estradiol. One interpretation of this finding is that higher circulating levels of estradiol leads to a higher increase in voluntary wheel running each day. However, females receiving both the 50 μg and 5 μg doses exhibit similar daily average running rates despite a 10-fold difference in circulating estradiol concentration (Figs. 2B, 3B, & 7B). Instead, increases in voluntary wheel running related more to the timing of estradiol replacement. This is evident in that estradiol-injected rats never exhibited an increase in wheel running on Day 1 regardless of estradiol dose. After Day 1, rats receiving both the 5 μg and 50 μg estradiol dose exhibited increased running, with overall non-rapid running rates steadily climbing with each subsequent day equivalently between experimental groups (Figs. 3B & 7B). Overall, there is strong evidence that the non-rapid actions of estradiol are changing the responsiveness of both non-rapid and rapid actions of estradiol on voluntary wheel running. The mechanism underlying this non-rapid effect of estradiol could potentially involve nuclear estrogen receptors. Nuclear estrogen receptor expression can be both downregulated and upregulated by repeated estradiol exposure dependent on tissue/cell type (Catanuto et al., 2009; Hatae et al., 2009; Patisaul et al., 1999; Saceda et al., 1988; Sharma and Thakur, 2006). Future work examining how estrogen receptor expression changes with estradiol replacement in relevant neural substrates could elucidate the mechanisms underlying differences in non-rapid estradiol responsiveness.

The second hypothesis posits that rats must display a high running rate in order to detect rapid actions of estradiol on voluntary wheel running. Both previous literature and our current study showed some support of this hypothesis. Neural and behavioral differences exist in rats that are high-runners compared to those that are low-runners and experiments that test mechanisms that modulate running are only observed in high-runners (Rhodes et al., 2005; Roberts et al., 2012; Roberts et al., 2014). These studies indicate that rats that have a high-running rate may be necessary to observe modulatory changes. One interpretation of our studies is that a rapid response to estradiol cannot be detected at either the 5 μg dose or during the first injection of estradiol because of a floor effect in which the motivation to run is too low. One way to test this hypothesis in future experiments is to elevate running rate via a method that does not employ estradiol, and then test whether a naïve response to a single estradiol injection at either dose and sex rapidly decreases voluntary wheel running.

We note that there are several alternative estradiol replacement methods available that could have potentially been employed. The injection paradigm used here was selected because many behavioral neuroscience studies use gonadectomy followed by 2–3 day estradiol injection procedures of various doses to induce a GNRH surge, or to mimic aspects of circulating estradiol levels similar to proestrus/estrus (Becker and Rudick, 1999; Cummings and Becker, 2012; Peterson et al., 2015; Scharfman et al., 2007). We replicated this literature by validating that daily injections of the lower 5 μg dose induced similar circulating estradiol levels to those measured in proestrus females. We note that the actual estradiol levels achieved throughout the injection paradigm are likely slightly higher than proestrus levels over a longer period of time, and that measured estradiol concentrations are likely not representative of peak levels for either the proestrus group or the 5 μg or 50 μg doses as blood samples were collected 24 hour post-injection. However, our studies indicated that there are still significant differences between intact proestrus females and 5 μg EB replacement females in terms of early-nocturnal period running rate despite similar circulating estradiol. In fact, our studies showed that the 50 μg EB replacement was better at replicating early dark phase running rates. Despite 50 μg EB having 10-fold higher circulating E2, rats receiving this dose never surpassed gonad-intact animal running rates. There are many differences between naturally cycling hormones in gonad-intact rats and injectable hormone replacements in gonadectomized rats, including dose, timing of hormone surges, hormones present (ie. progesterone), and potential endocrine compensation that occurs after surgical gonadectomy. For example, progesterone-only injections have previously shown to decrease voluntary wheel running (Rodier, 1971). Estradiol priming increases neuroprogesterone production (Micevych et al., 2008) so it cannot be ruled out that neuroprogesterones are playing a role in the rapid-estradiol responsiveness observed in this study. Future studies testing the role of motivation will be able to further untangle these variables in naturally cycling rats.

Notably, we report sex-responsive effects of both non-rapid and rapid actions of estradiol on voluntary wheel running. We are defining sex-responsive effects as those that are similar in direction and timing in females and males but feature a sex difference in the robustness of the effect. We propose this “sex-responsive” definition as an extension upon previous laboratory groups who have defined different categories of sex differences based on factors such as timing, permanence, mechanism, and functional purpose (Arnold, 2017; De Vries, 2004; Joel and McCarthy, 2017). Specifically, regarding voluntary wheel running, we show that males have the capacity for presenting female-typical voluntary wheel running both rapidly and non-rapidly to 50 μg of EB. For non-rapid estradiol actions, males required the 50 μg EB dose to increase voluntary wheel running (Fig. 3B) where 5 μg EB was sufficient in females (Fig. 7B). It is possible that if males continued to receive repeated doses of 5 μg EB for longer than 4 days, then voluntary wheel running would more resemble female-typical responses, but this would need to be tested. For rapid estradiol actions, both males and females required the 50 μg to display decreases in voluntary wheel running dependent on prior daily estradiol injections (Fig. 5D&E). Females showed this decrease sooner and more strongly than males. These findings add to previous literature that prior estradiol exposure influences future estradiol responsiveness in both sexes (Becker and Rudick, 1999; Meitzen et al., 2012; Micevych and Meisel, 2017). Two potential non-competing alternative hypotheses are sex differences in estrogen receptor expression in brain loci controlling voluntary wheel running or sex differences in bioavailability of estradiol via steroid binding globulins and metabolism. Overall, these findings reaffirm that ovarian-sourced estradiol has an activational role in generating a sex difference in voluntary wheel running and provide further nuance that the different wheel running behaviors between males and females is dependent upon the analyzed temporal domain and estradiol-responsiveness.

There is precedence for considering rapid and non-rapid actions of estradiol congruently on animal behavior (Kow and Pfaff, 2004; Rainville et al., 2015). The dual action of estrogen hypothesis (Cornil et al., 2015) asserts that rapid estradiol actions control motivational aspects of behaviors whereas non-rapid estradiol actions control consummatory or performance aspects of behaviors. These aspects are often defined as separate behaviors. Here, we present evidence for both rapid and non-rapid estradiol actions on a single behavior, voluntary wheel running, further extending this hypothesis to consider how non-rapid and rapid estradiol actions interact in a single motivated behavior. One interpretation of this extension is that a single behavior can exhibit both motivational and consummatory/performance aspects and is perhaps controlled by estradiol action at different neural loci within the relevant circuit. A second interpretation is that it is possible for a single type of behavior, in this case a motivation-relevant behavior, to be modulated by both rapid and non-rapid estrogen action (Cross and Roselli, 1999). Whichever interpretation is favored, we believe this study demonstrates the broad spectrum of estradiol signaling by showing that a single hormone signal can induce bidirectional effects on a single behavior dependent on the temporal domain. Furthermore, estradiol's effect may be masked or uncovered by the present hormone state of the animal as well as the sex and/or hormonal history. Overall, this study demonstrates the utility of considering sex and hormone temporal interactions when assessing hormonal neuromodulatory mechanisms and behavioral outcomes.

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A.A.K. designed the experiments, collected data, analyzed data, and contributed to drafts the manuscript. S.P. collected data that contributed to this manuscript. H.P. designed experiments, provided behavioral analysis software and running wheels, and contributed to drafts of the manuscript. J.M. designed the experiments and contributed to drafts of the manuscript. We would like to thank the Biological Resources Facilities of North Carolina State University for assistance and animal care resources, David Dorris for technical support, and Natalie Truby for assistance on studies. We used Biorender.com to design the graphical abstract.

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