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The estrogenic reduction in water intake stimulated by dehydration involves estrogen receptor alpha and a potential role for GLP-1

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ABSTRACT

It is well documented that estrogens inhibit fluid intake. Most of this research, however, has focused on fluid intake in response to dipsogenic hormone and/or drug treatments in euhydrated rats. Additional research is needed to fully characterize the fluid intake effects of estradiol in response to true hypovolemia. As such, the goals of this series of experiments were to provide a detailed analysis of water intake in response to water deprivation in ovariectomized female rats treated with estradiol. In addition, these experiments also tested if activation of estrogen receptor alpha is sufficient to reduce water intake stimulated by water deprivation and tested for a role of glucagon like peptide-1 in the estrogenic control of water intake. As expected, estradiol reduced water intake in response to 24 and 48 h of water deprivation. The reduction in water intake was associated with a reduction in drinking burst number, with no change in drinking burst size. Pharmacological activation of estrogen receptor alpha reduced intake. Finally, estradiol-treatment caused a leftward shift in the behavioral dose response curve of exendin-4, the glucagon like peptide-1 agonist. While the highest dose of exendin-4 reduced 10 min intake in both oil and estradiol-treated rats, the intermediate dose only reduced intake in rats treated with estradiol. Together, this series of experiments extends previous research by providing a more thorough behavioral analysis of the anti-dipsogenic effect of estradiol in dehydrated rats, in addition to identifying the glucagon like peptide-1 system as a potential bioregulator involved in the underlying mechanisms by which estradiol reduces water intake in the female rat.

1. Introduction

The estrogenic involvement in the control of water intake has been appreciated since the late 1970s. Behavioral studies have demonstrated that unstimulated water intake is reduced on the day of estrus and the loss of circulating ovarian hormones via ovariectomy induces a transient increase in daily water intake in rats, that can be restored by exogenous estradiol treatment [1–7]. A direct inhibitory effect of estradiol on water intake, however, is difficult to conclude from these studies, due to the anorexigenic effect of estradiol and the prandial nature of rodents [8–11]. Nevertheless, experiments using hormones or drugs that stimulate water intake, such as AngII or isoproterenol, also reveal the anti-dipsogenic effect of estradiol in the absence of food intake [12–20]. More recent work has demonstrated that the fluid intake effects of estradiol are not limited to just reducing intake. In paradigms of reduced water drive, i.e. unstimulated drinking in the absence of food, estradiol enhances water intake, revealing the hormone's bidirectional role in the controls of fluid intake [6,21]. These dual roles of estradiol likely act in concert to defend fluid homeostasis, maintenance of which is critical for survival, however, the underlying neuronal and molecular mechanisms by which estradiol controls water intake are unknown.

Most of the research on the anti-dipsogenic effects of estradiol has been conducted in euhydrated rats treated with thirst driving stimuli. While useful information has been gained from these studies, limited research has been conducted in hypovolemic rats. It is unclear how generalizable the results obtained using AngII or isoproterenol are to drinking in response to dehydration because the acute hypertension caused by AngII in euhydrated rats dampens the drinking response [22,23]. To our knowledge only three previous studies examined the effect of estradiol on water-deprivation induced drinking and two of these studies used chronic daily injections of estradiol, which does not mimic the cyclic release of the hormone [14,19,20]. Although all of these studies demonstrated that estradiol decreases water intake, important aspects of the behavioral change, including the time course of intake and

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drinking microstructure have not been explored. Furthermore, studies aimed at understanding the underlying mechanisms by which estradiol controls water intake have provided little insight beyond ruling out potential targets and again, have primarily been conducted in euhydrated rats treated with AngII or isoproterenol [13,20,24,25].

The goals of this series of experiments were to provide a more comprehensive understanding of the estrogenic-mediated reduction in water intake in response to dehydration in the female rat. To do so, we tested the hypothesis that estradiol reduces water intake in response to 24 and 48 h water deprivation via discrete drinking microstructure changes in burst number and/or burst size. We also examined whether estrogen receptor alpha (ERα) activation is involved in the anti-dipsogenic effects of estradiol in response to water deprivation. ERα was targeted because activation of this receptor reduces AngII-stimulated water intake [13]. Finally, we tested if estradiol alters the fluid inhibitory effects of the glucagon like peptide-1 (GLP-1) agonist, exendin-4, with the goal of identifying a bioregulator system that may be involved in the mechanism by which estradiol inhibits water intake.

2. Methods

2.1. Animals and housing

Adult female Long Evans rats (Envigo), approximately 80 days of age at the start of the experiments, were used throughout. Rats were housed two per cage upon arrival into the facility, but after surgery all rats were singly housed in modified shoebox cages. Rats had ad libitum access to food (Teklad 2018; Envigo) and tap water unless otherwise noted. Body weights were recorded Monday-Fridays. The temperature- and humidity- controlled colony rooms were maintained on a 12:12 h light-dark cycle (lights on at 0700 h). All experimental procedures were approved by the Animal Care and Use Committee at the University of Kentucky. The handling and care of the rats was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Surgery

All rats were ovariectomized (OVX) approximately one week after arriving at the facility. Briefly, rats were anesthetized with isoflurane and given 5 mg/kg carprofen (subcutaneously (sc); Henry Schein). Female rats were then bilaterally OVX using an intraabdominal approach, as previously described [26]. At the end of the surgy, rats received 5 ml of sterile 0.9 % saline (sc) and 24 h later received a second injection of 5 mg/kg carprofen. Rats were given at least one week to recover from surgery before the experimental procedures began.

2.3. Intake and lick measures

Volumetric water intakes (ml) were measured at the start and end of the testing period. The distribution of drinking throughout the testing period was measured using a custom designed contact lickometer (University of Kentucky, College of Arts and Sciences Electronics Shop) that connected to modified shoebox cages containing two external lick blocks (University of Kentucky, College of Arts and Sciences Machine Shop). Bottle spouts were behind an electronically isolated metal plate with a 3.175 mm-wide opening, through which the rat needed to lick to reach the spout, minimizing non-tongue contacts (i.e., paw) with the spout. The lickometer interfaced with a computer using an integrated USB digital I/O device (National Instruments, Austin, TX) to record individual time-stamped licks, after which the data were processed in Excel. Time stamped licks allow for the analysis of (1) intake as a function of time and (2) drinking microstructure, both of which can also be analyzed in time bins of varying sizes. A drinking (lick) burst was defined as at least 2 licks with an inter-lick-interval (ILI) of no more than 1 s and burst size was defined as the number of licks within a burst, as previously described [27]. Drop sizes (total volume/total number of licks) were calculated to confirm there was no excessive bottle leakage (drop sizes > 6 μl) or clogged bottles (drop sizes < 3 μl). Volumetric calculations prior to the end of the drinking test (Expt. 1) were determined by multiplying the drop size by cumulative licks at the specific timepoint.

2.4. Experimental design

2.4.1. Experiment 1. Does estradiol reduce water intake stimulated by 24 h water deprivation by a change in burst number or burst size?

Eleven female Long Evans rats were treated sc with 10 μg estradiol benzoate (EB, Sigma) or peanut oil control (0.1 ml) at 0900 h on days 1 and 2. On day 3, water bottles were removed at 0900 h. Twenty-four hours later (day 4), food was removed from the cages and rats were given pre-weighted water bottles. Water intake and licks were measured for 2 h. The following week, rats received the opposite hormone treatment. This hormone replacement paradigm and testing protocol were based on a previous report demonstrating that acute EB treatment reduced water intake stimulated by 24 h water deprivation in OVX rats [14].

2.4.2. Experiment 2. Does activation of ERα reduce water intake simulated by 24 h water deprivation?

The rats from Experiment 1 were subsequently used in Experiment 2 ($n = 11$). On day 1, water bottles were removed from the cages at 1300 h. On day 2, rats were treated sc with 200 μg PPT (ERα agonist, Tocris) or 50 % DMSO control (0.1 ml) at 0900 h. At 1300 h, food was removed from the cages and rats were given pre-weighted water bottles and intake and licks were measured for 40 min (see Results Experiment 1). The following week rats received the opposite drug treatment. The time course and drug dose chosen were based on our previous studies with PPT, which has a more rapid onset of action compared to EB [13,28].

2.4.3. Experiment 3. Does estradiol reduce intake when rats are water deprived for 48 h?

Twelve experimentally naive rats were used in this experiment. The protocol was identical to Experiment 1 except that water was removed just after hormone treatment on day 2, instead of day 3, resulting in 48 h of water deprivation.

2.4.4. Experiment 4. Does estradiol augment exendin-4's inhibitory effect on water intake?

Thirteen experimentally naive rats were used in this experiment. Rats were treated sc with 10 μg EB or oil (0.1 ml) at 0900 h on days 1 and 2. On day 3, water bottles were removed. On day 4, food was removed from the cages and rats were injected intraperitoneal with 0, 0.1, 1.0, or 3.0 μg/kg exendin-4 (Sigma) just prior to returning water at 0900 h, after which intake and licks were measured for 40 min. Testing occurred once a week for eight weeks until all rats received all drug doses and hormone combinations. These drug doses were chosen based on past research [29].

2.5. Data analysis

Data are presented as means + SEM throughout. The statistical package Statistica was used to analyze all data. Data normality was verified with a Shapiro-Wilk test.

Paired *t*-tests were used to analyze water intake volume (ml), burst number, and burst size (licks/burst) for Experiments 1–3. A Wilcoxon matched pairs test was used when data failed the normality test (Expt. 1 burst size, Expt. 2 burst number, Expt. 3 vol and burst number). A two-factor repeated measures ANOVA (hormone/drug X time) was used to analyze non-cumulative licks during the test sessions for Expt. 1–3. A two-factor repeated measures ANOVA (drug X hormone) was used to analyze water intake, burst number, and burst size and a three-factor

repeated measures ANOVA (drug X hormone X time) was used to analyze non-cumulative licks for Expt. 4. Tukey's post hoc tests were used to follow all significant main and interactive effects.

3. Results

3.1. Experiment 1. Does estradiol reduce water intake stimulated by 24 h water deprivation by a change in burst number or burst size?

While previous research has demonstrated that estradiol reduces water intake stimulated by 24 h water deprivation in OVX rats [14,19,20], the underlying drinking microstructure changes associated with the reduced intake are unknown. Furthermore, a detailed time course of this effect has not been conducted. Water intake for the entire 2 h test was first analyzed. Water intake was significantly reduced after EB treatment ($t_{(9)} = 2.96$, $p < 0.05$; data not shown). Next, non-cumulative licks across the 2 h test period were examined in 10 min bins, which demonstrated that licks were made primarily within the first 10 min of the test, with drinking ending by the 40-minute mark in both EB and oil-treated rats ($F_{(11,99)} = 141.10$, $p < 0.001$; Fig. 1A). There was also an interaction between hormone and time ($F_{(11,99)} = 3.642$, $p < 0.001$). During the first 10 min of the drinking test, rats treated with estradiol had significantly less licks than oil-treated rats ($p < 0.0005$). To determine if there was a more discrete time frame during the first 10 min where EB reduced intake, licks were analyzed in 1-, 2-, and 5-min bins during this time. However, regardless of the bin size, a main effect of hormone was detected ($F_{(1,9)} = 10.68$, $p < 0.001$) but no interaction between hormone and time ($F_{(1,9–36–81)} = 0.93 – 1.29$, $p = n.s.$; 1 min bins presented in Fig 1B).

Due to the drinking being limited to the first 40 min of intake, the remainder of the intake analyses (and testing in Expt. 2–4) were limited to this timeframe. As expected, water intake during the 40 min timeframe was significantly reduced after EB treatment ($t_{(9)} = 8.58$, $p < 0.001$; Fig. 1C). Analysis of licks in 10 min bins during this timeframe revealed a main effect of time ($F_{(3,27)} = 174.22$, $p < 0.001$), and a main effect of hormone ($F_{(1,9)} = 17.57$, $p < 0.01$), but the interaction just failed to reach significance ($F_{(3,27)} = 2.77$, $p = 0.06$; Fig. 1D). Regardless of time, EB treatment reduced licks for water and regardless of hormone treatment, licks for water were greatest during the first 10 min bin. Analysis of the drinking microstructure during the 40 min period revealed that EB significantly reduced burst number ($t_{(9)} = 3.65$, $p < 0.01$; Fig. 1E), but had no effect on burst size ($Z_{(9)} = 0.96$, $p = n.s.$; Fig. 1F). One rat was excluded from analysis due to excessive bottle leakage.

3.2. Experiment 2. Does activation of ERα reduce water intake simulated by 24 h water deprivation?

ERα underlies the anti-dipsogenic effect of estradiol associated with AngII-stimulated intake [13]. Whether this extends to other stimuli that robustly drive water intake is unclear. PPT treatment just failed to significantly reduce water intake stimulated by 24 h water deprivation ($t_{(10)} = 1.87$, $p = 0.09$; Fig. 2A). Analysis of licks in 10 min bins revealed a main effect of time ($F_{(3,30)} = 197.73$, $p < 0.001$), and an interaction between drug and time ($F_{(3,30)} = 6.72$, $p < 0.01$; Fig. 2B). Again, licks for water were greatest during the first 10 min bin. During this time, PPT-treatment significantly reduced licks for water ($p < 0.05$). Because the anti-dipsogenic effect of PPT was only detected during the first 10 min bin, drinking microstructure analysis was conducted during this timeframe. Surprisingly, PPT treatment had no effect on either burst number ($Z_{(10)} = 0.36$, $p = n.s.$; Fig. 2C), or burst size ($t_{(10)} = 0.21$, $p = n.s.$; Fig. 2D).

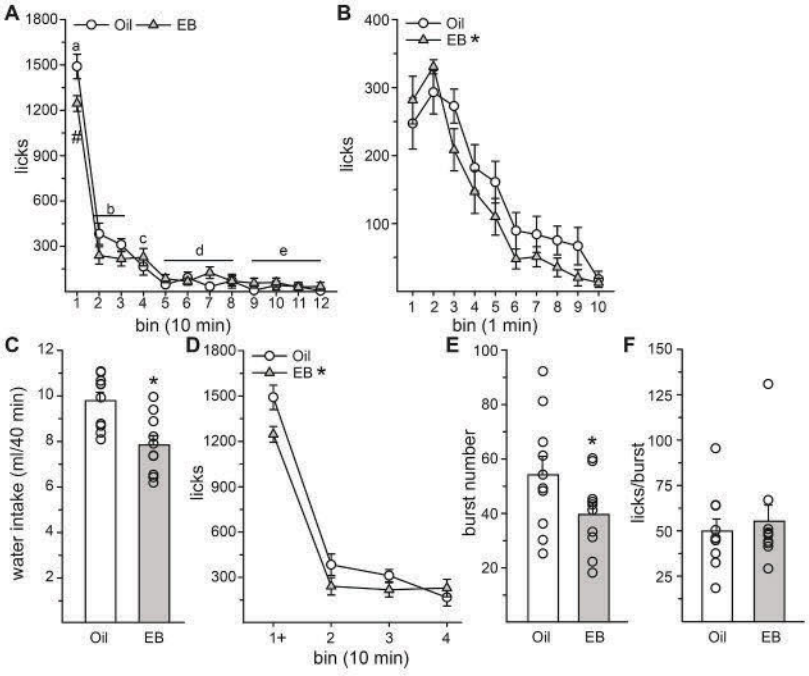

Fig. 1. EB treatment reduced water intake stimulated by 24 h water deprivation. (A) Analysis of licks in 10 min bins revealed that intake lasted for 40 min post water bottle access. Intake during the first 10 min was significantly greater than intake at all other timepoints. During this time, EB-treated rats had less licks than oil-treated rats and (B) a 1 min bin size analysis did not reveal any additional time specific changes in intake. (C) 40 min water intake was reduced when rats were treated with EB. (D) Licks for water were greatest during the first 10 min (bin 1) of testing. Regardless of time, EB treatment reduced licks, compared to Oil treatment. (E) EB treatment significantly reduced burst number, (F) but had no effect on burst size (licks/burst). #Less than oil during bin 1. [a]Greater than all other time bins. [b]Less than bin 1, but greater than bins 5–12. [c]Less than bin 1, but greater than bins 9–12. [d]Less than bins 1–3. [e]Less than bins 1–4. *Less than oil, $p < 0.01$. +Greater than licks during bins 2–4.

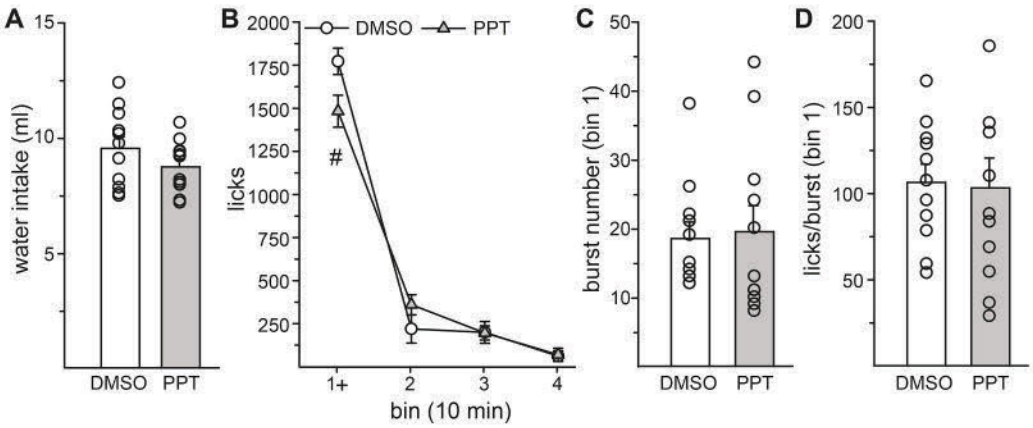

Fig. 2. Activation of ERα reduced water intake stimulated by 24 h water deprivation. (A) There was no significant difference in 40 min water intake between control and PPT treatment. (B) Licks for water were greatest during the first 10 min (bin 1) of testing. During this time, PPT treatment significantly reduced licks, compared to DMSO treatment. (C) PPT treatment had no effect on burst number, (D) nor reduced burst size (licks/burst). +Greater than licks during bins 2–4. #Less than 10 min oil group, $p < 0.01$.

3.3. Experiment 3. Does estradiol reduce intake when rats are water deprived for 48 h?

It is unclear if prolonged dehydration, or the level of hypovolemia, augments the anti-dipsogenic effect of estradiol, therefore intake after 48 h of water deprivation was tested. Water intake was significantly reduced after EB treatment ($Z_{(11)} = 3.06$, $p < 0.001$; Fig. 3A). **Analysis of licks in 10 min bins revealed a main of time ($F_{(3,33)} = 220.65$, $p < 0.001$),** a main effect of hormone ($F_{(1,11)} = 30.34$, $p < 0.001$), and an interaction between time and hormone ($F_{(3,33)} = 3.76$, $p < 0.05$; Fig. 3B). **Regardless of time, EB treatment reduced licks for water and regardless of hormone treatment, licks for water were greatest during the first 10 min bin. During the first 10 min bin, EB treatment reduced licks for water ($p <$ 0.01). EB significantly reduced burst number ($Z_{(11)} = 2.67$, $p < 0.01$;** Fig. 3C), **but had no effect on burst size ($t_{(11)} = 0.69$, $p = n.s.$;** Fig. 3D).

3.4. Experiment 4. Does EB augment exendin-4's inhibitory effect on water intake?

It is unclear which satiety factors estradiol interacts with to reduce water intake. We, therefore, tested for an interaction between EB and the GLP-1 receptor agonist exendin-4. Water intake was influenced by a main effect of drug ($F_{(3,36)} = 92.85$, $p < 0.001$), and a main effect of hormone ($F_{(1,12)} = 23.87$, $p < 0.001$), but no interaction between drug and hormone ($F_{(3,36)} = 0.50$, $p = n.s.$; Fig. 4A). **As expected, EB treatment reduced water intake. Water intake after treatment with 1.0 μg/kg exendin-4 was significantly reduced compared to intake after treatment with 0 and 0.1 μg/kg exendin-4 ($p < 0.01$). Intake after treatment with 3.0 μg/kg exendin-4 was less than intake after treatment with all other doses. Analysis of licks in 10 min bins revealed a main effect of time ($F_{(3,36)} = 363.32$, $p < 0.001$),** a main effect of drug ($F_{(3,36)} = 68.58$, $p <$ 0.001), and a main effect of hormone ($F_{(1,12)} = 14.30$, $p < 0.01$). Interactions between drug and time ($F_{(9108)} = 15.00$, $p < 0.001$), between hormone and time ($F_{(3,36)} = 4.88$, $p < 0.01$), and between drug, time and hormone ($F_{(9108)} = 2.11$, $p < 0.05$), were also detected (Table 1). Fig. 4B graphs the licks during the first 10 min bin, where the three-way interaction between drug, time, and hormone was detected. Again, regardless of drug treatment, EB reduced licks and regardless of hormone treatment, the higher two doses of exendin-4 reduced licks in a dose dependent manner ($p < 0.005$). During this 10 min bin, EB produced a leftward shift in the dose-response curve to exendin-4. Licks after treatment with 1.0 μg/kg exendin-4 were only reduced, compared to vehicle, when rats were treated with EB ($p < 0.05$). However, the 3.0 μg/kg dose reduced licks regardless of hormone treatment ($p < 0.001$). Burst number was influenced by a main effect of drug ($F_{(3,36)} = 19.97$, p < 0.001), but not hormone ($F_{(1,12)} = 1.17$, $p = n.s.$), nor an interaction between drug and hormone ($F_{(3,36)} = 0.30$, $p = n.s.$; Fig. 4C). **Burst number was significantly reduced after treatment with 1.0 and 3.0 μg/kg**

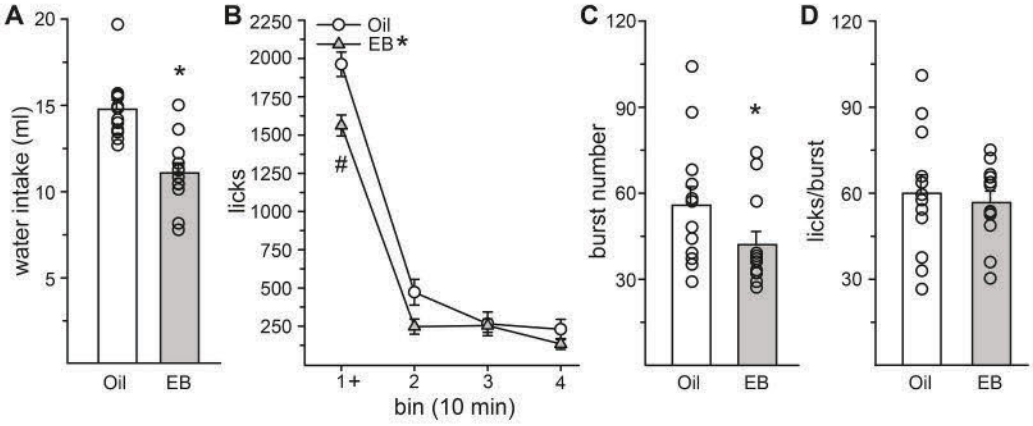

Fig. 3. EB treatment reduced water intake stimulated by 48 h water deprivation. (A) 40 min water intake was reduced when rats were treated with EB. (B) Licks for water were greatest during the first 10 min (bin 1) of testing. EB treatment reduced licks, compared to Oil treatment, specifically within the first 10 min (bin 1) of testing (C) EB treatment significantly reduced burst number, (D) but had no effect on burst size (licks/burst). *Less than oil, $p < 0.01$. +Greater than licks during bins 2–4. #Less than 10 min oil group, $p < 0.01$.

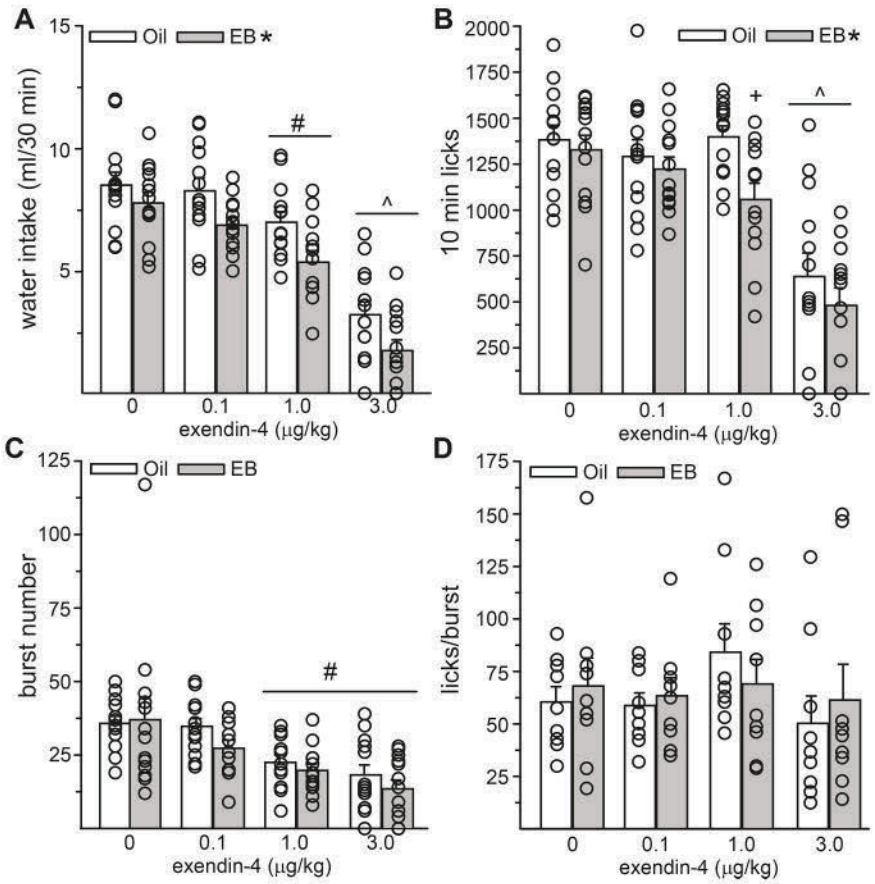

Fig. 4. EB treatment produced a leftward shift in the dose response curve to exendin-4. (A) 40 min water intake was reduced by EB treatment. Water intake was reduced by treatment with 1.0 but not 0.1 μg/kg exendin-4 and intakes were further reduced by treatment with 3.0 μg/kg exendin-4. (B) Licks for water during the first 10 min were reduced by EB treatment. While the 1.0 μg/kg failed to reduce water intake in oil treated rats, there was a significant reduction in water intake when rats were treated with EB. The 3.0 μg/kg reduced licks in both oil and EB-treated rats. (C) 1.0 and 3.0 μg/kg exendin-4 significantly reduced burst number but (D) had no effect on burst size (licks/burst). *Less than oil, $p < 0.01$. #Less than 0 and 0.1 μg/kg exendin-4, $p < 0.001$. ^Less than 0, 0.1, and 1.0 μg/kg exendin-4, $p < 0.001$. +Less than vehicle treated rats, $p < 0.05$.

Table 1
Licks in 10-minute bins during the test period.

Drug Dose (μg/kg)	Bin 1[%] (0–10 min)		Bin 2 (10–20 min)		Bin 3 (20–30 min)		Bin 4 (30–40 min)	
	Oil	EB*	Oil	EB*	Oil	EB*	Oil	EB*
0	**1382.31 ± 78.83**	**1327.46 ± 79.52**	**366.85 ± 23.17**	**281.23 ± 60.97**	**233.08 ± 42.47**	**223.69 ± 33.50**	**187.46 ± 42.73**	**207.92 ± 47.21**
0.1	**1292.00 ± 90.99**	**1222.46 ± 66.26**	**281.08 ± 52.86**	**288.38 ± 54.22**	**328.08 ± 43.20**	**139.77 ± 19.39**	**148.32 ± 42.00**	**210.38 ± 51.47**
1[#]	1398.23 ± 59.21	1057.23 ± 89.04[+]	119.15 ± 39.61	77.46 ± 33.82	91.85 ± 42.89	92.92 ± 41.50	26.23 ± 18.53	44.38 ± 24.81
3[^]	638.31 ± 128.01[+]	480.77 ± 94.96[+]	27.00 ± 15.86	3.38 ± 3.38	15.15 ± 14.50	0.00 ± 0.00	15.00 ± 14.11	13.69 ± 10.52

[%] Main effect time: Greater than licks during bins 2–4, $p < 0.001$.
[*] Main effect hormone: Less than oil, $p < 001$.
[#] Main effect dose: Less than licks after 0 and 0.1 μg/kg exendin, $p < 0.001$.
[^] Main effect dose: Less than licks after 0, 0.1, and 1 μg/kg exendin, $p < 0.001$.
[+] Interaction time, hormone, and dose: Significantly less licks compared to Oil/EB 0 μg/kg exendin, $p < 0.01$.

exendin-4, compared to 0 or 0.1 μg/kg exendin-4. Burst size was not influenced by drug ($F_{(3,27)} = 1.73$, $p = n.s.$), hormone ($F_{(1,9)} = 0.03$, $p = n.s.$), nor an interaction between drug and hormone ($F_{(3,27)} = 1.46$, $p = n.s.$; Fig. 4D). **Burst size analysis was limited to 10 rats due to 4 rats making no licks, and therefore having 0 bursts, after treatment with 3.0 μg/kg exendin-4. When there are 0 bursts, the burst size cannot be defined (licks/0 = undefined).**

4. Discussion

This report adds to our understanding of how estradiol reduces water intake in female rats from both a behavioral and mechanistic level. These data demonstrate that changes in burst number, but not burst size, underlie the anti-dipsogenic effect of estradiol in response to water deprivation. In addition, activation of ERα reduced licks for water stimulated by 24 h water deprivation. While similar results have been

observed when examining water intake stimulated by central treatment with AngII [12,13], these findings are an important extension of that work because they demonstrate that those observations generalize to the dehydrated state, as opposed to paradigms that only model aspects of dehydration. Furthermore, this study identifies the GLP-1 system as a potential target that estradiol may augment to reduce water intake. Future studies can focus on this neuropeptide and its receptor system to elucidate the neuronal mechanisms underlying the fluid intake effects of estradiol.

While the anti-dipsogenic effect of estradiol has been well documented, to our knowledge only three previous studies examined the effect of estradiol on water-deprivation induced drinking [14,19,20]. These reports, however, only examined volumetric changes in intake. Fully characterizing the behavioral changes (drinking microstructure, detailed time course) by which estradiol reduces water intake in this paradigm is a critical step necessary for elucidating the underlying neuronal mechanism. Most of our understanding of the fluid intake effects of estradiol come from studies that stimulate intake with AngII or isoproterenol in euhydrated animals. As such, drinking in these paradigms occurs under very different physiological conditions, as animals need body fluids and have low blood pressure, in the dehydrated state, but have sufficient body fluids and normal blood pressure, in the euhydrated state. This mismatch between the exogenic dipsogenic stimuli and normal blood volume confounds the drinking response. In the euhydrated rat, instead of restoring normal blood pressure that would be low in the dehydrated state, the pressor response caused by AngII creates acute hypertension, which itself dampens water intake [22,23]. Furthermore, two of these past studies used chronic estradiol replacement, which does not mimic the cyclic nature of the hormone's release [19,20]. We used the same acute hormone replacement paradigm as Krause et al. [14], which closer mimics the phasic release of estradiol [30]. Future studies, however, should examine the drinking response after ER antagonist treatment in intact cycling rats for a more complete understanding of the estrogenic inhibition of water intake in relationship to normal hypothalamic-pituitary-gonadal physiology.

As expected, estradiol reduced water intake after 24 h water deprivation. As an extension of previous work, our results show that drinking was greatest during the first 10 min of water access and concluded by 40 min. The previous reports limited intake measures to either 2 h post water access [14,20] or in 1 h intervals (cumulative intakes) for 3 h [19]. During the 40 min of water consumption, the total number of drinking bursts was reduced by estradiol treatment, but the average burst size (licks/burst) was not changed. This suggests that changes in post-ingestive feedback signals underlie the fluid intake effects of estradiol [31,32], which complements previous research demonstrating that changes in burst number are associated with the estrogenic reduction of AngII-stimulated water intake [12]. Estradiol has a bidirectional effect on water intake and can increase water intake in euhydrated rats when food is removed [6,21]. Under these circumstances, the increase in water intake is also driven by changes in burst number [6]. Together, this suggests that regardless of the direction of the change in intake, estradiol modulates post-ingestive signals to alter water intake in the female rat. One caveat of this experiment is rats retained food during the water deprivation period. Although not measured here, serum osmolality was likely increased [33] and therefore the drinking response was driven by both hypovolemic and osmotic thirst. Since it is well reported that estradiol does not influence drinking in response to intracellular dehydration [4,14,17,19,34], we attribute the reduction in intake to the hypovolemic thirst, although caution should be taken with this interpretation.

Next, we determined if activation of ERα is sufficient to reduce water intake stimulated by 24 h water deprivation. Although total volumetric intake at 40 min was not significantly reduced after agonist treatment ($p = 0.09$), analysis of licks as a function of time revealed more discrete changes. During the first 10 min of intake, when intake was most pronounced, rats treated with the ERα agonist made fewer licks than when control treated. Given the different pharmacokinetic properties between estradiol benzoate and PPT and the well reported differences in the time course of action [28,35], it is not necessarily surprising that the behavioral effect of PPT was shorter acting than EB. What is important, however, is that PPT did reduce licks during the drinking test. This is in line with previous research demonstrating that ERα underlies the anti-dipsogenic effect of estradiol associated with AngII-stimulated and unstimulated intake [13]. Surprisingly, we found no change in the drinking microstructure during this time. Our previous research demonstrated that the PPT-induced reduction in water intake after AngII-treatment is mediated by changes in burst number, similar to estradiol, and not burst size [13]. It is unclear why no change was detected in this study and future studies will be needed to understand the microstructure changes underlying PPT-mediated reductions in water intake stimulated by dehydration. Perhaps a shorter time interval between agonist treatment and water access would have revealed the change in drinking microstructure or a longer period of deprivation (48 h) to increase baseline intake. One limitation of the present research is that we did not rule out the involvement of ERβ or GPER-1 in the anti-dipsogenic effect of estradiol in response to 24 h water deprivation. Because our previous research has indicated that these receptors are not involved in the inhibitory effects of estradiol on water intake [13,36], we chose to focus on just ERα in this series of experiments. One additional consideration in interpreting these results is that dehydration alters ER expression in thirst relevant brain regions in male rats [37,38]. Whether similar changes occur in female rats, specifically OVX females, is unknown and an interesting avenue for future research.

Most studies on the fluid intake effects of estradiol have been conducted in euhydrated animals, therefore, it is unclear if the degree of hypovolemia can augment or prevent the anti-dipsogenic effect of estradiol. We, therefore, examined the effect of estradiol on water intake after 48 h of water deprivation. Similar to our findings in response to 24 h water deprivation, estradiol treatment reduced water intake, licks, and burst number, with no change in burst size. This demonstrates that the anti-dipsogenic effect of estradiol remains intact even at this more severe level of dehydration [39–41]. While we did not design the experiments to explicitly compare intakes between the 24 h and 48 h deprivation periods, the percent decreases in water intake after EB treatment was not different between experiments (24 $h = -20.02\ \% \pm 2.39$ vs 48 $h = -25.18\ \% \pm 2.48$, $p = n.s.$), however, as expected intake was greater after 48 h of water deprivation in oil treated rats (24 $h = 9.76$ ml $\pm$ 0.37 vs 48 $h = 14.78$ ml $\pm$ 0.53, $p < 0.001$).

Despite the anti-dipsogenic effects of estradiol first being reported in the 1970s, there is still very little information regarding estradiol's neuronal and molecular targets that drive these changes in water intake. While past studies have focused on the renin-angiotensin system, a direct effect on water intake is unlikely as estradiol does not alter plasma renin activity, circulating AngII levels, hypothalamic-septal mRNA expression of AT2R, ACE, renin, AGT or angiotensin type 1 receptor once body weight changes are taken into account [13,20,24,42,43]. Increased vasopressin could help compensate for reduced water intake. In fact, there is an increase in plasma vasopressin in response to 24 h water deprivation in estradiol, compared to oil-treated, rats [20]. Centrally, there is increase in AngII-induced c-fos expression in PVN vasopressin neurons after estradiol treatment, and there is an increase in water deprivation induced c-fos expression in PVN and SON vasopressin neurons after estradiol treatment [20,25]. The effect of estradiol on urine volume, however, has not been measured in response to AngII-treatment. Furthermore, in water deprived rats, there is no difference in urine volume between oil and EB-treated rats. After rehydration, urine volume is greater in EB, compared to oil, -treated rats, not reduced as would be predicted by the increases in plasma vasopressin [20]. Finally, in euhydrated rats, there is no change in urine excretion across the estrous cycle [3]. Therefore, it is unclear how any estradiol-mediated change in vasopressin influences water intake. Our current and previous reports consistently demonstrate that changes in

burst number underlie both the inhibitory and stimulatory effects of estradiol on water intake. As a change in burst number reflects changes in post-ingestive feedback signals, it is logical to test for interactions between estradiol and bioregulators involved in water satiation.

The bioregulator GLP-1, while best identified for its role in insulin regulation, has an inhibitory effect on both water, saline, and food intake [44,45]. The fluid inhibitory effect of GLP-1 is associated with changes in burst number, and the anorexigenic effect of GLP-1 is also associated with changes in satiety, as observed by reductions in meal size [46–48]. In addition, GLP-1 is involved in the anorexigenic effect of estradiol [47,49]. Because our past and previous findings indicate that estradiol reduces water intake through changes in burst number (suggesting changes in post-ingestive/satiety signals), and the previously identified interactions between estradiol and GLP-1 on food intake, we targeted the GLP-1 system here. First, in support of previous research, we observed dose-related decreases in water intake, that were mediated by changes in burst number, after treatment with exendin-4, the GLP-1 agonist. Although no interactions between estradiol and exendin-4 treatment were observed in total volume consumed, there were interactions detected during the first 10 min of intake, when intake is greatest. During this time, estradiol produced a leftward-shift in the dose-response curve to exendin-4. During this time, only the 3.0 μg/kg dose of exendin-4 reduced licks for water in oil-treated rats, however, when treated with estradiol, both the 1.0 and 3.0 μg/kg doses of exendin-4 reduced licks. This mimics the finding that estradiol makes female rats more sensitive to the anorexigenic effect of exendin-4 [47]. Future studies should determine if exogenous GLP-1 is involved in the anti-diposgenic effect of estradiol.

Current evidence supports the role of brain derived GLP-1 being more critical for the fluid intake effects, than peripheral GLP-1. For example, water deprivation increases NTS, but not ileum, proglucagon mRNA expression and there is no change in circulating GLP-1 in response to water deprivation or rehydration [50]. If estradiol enhances the fluid satiating properties of the GLP-1 system, the effect is likely mediated directly in the brain. In support of this, ERα expressing neurons are in the NTS, where the central GLP-1 neurons are located [51–53]. ERα and GLP-1 receptors are both expressed in areas of the brain that control fluid intake, for example in the MnPO and OVLT, although expression of both receptors is widespread throughout the central nervous system [51,54]. GLP-1 induced c-fos expression is enhanced in estradiol treated rats in the PVN, another brain area critical for fluid balance [47]. ERα, however, is not expressed in the PVN, so any changes in this brain area might be downstream of direct effects of estradiol [51]. Future studies should determine if estradiol increases proglucagon expression in the NTS and/or increases expression of the GLP-1R in nuclei critical for water intake.

A better understanding of the neuronal and molecular mechanisms by which estradiol inhibits water intake is critical for elucidating how fluid balance is controlled in females. Despite decades of research on this topic, the central targets of estradiol that mediate the anti-dipsogenic effect are still unclear. This series of experiments suggests that the GLP-1 system is a logical target for investigation, as estradiol treatment altered the dose response curve and made rats more sensitive to lower doses of the GLP-1 agonist. This series of experiments also demonstrated that changes underlying the anti-dipsogenic effect of estradiol in response to water deprivation, such as decreases in burst number and the involvement of ERα, mimic changes observed with other paradigms of stimulated water intake. While most literature has examined the anti-dipsogenic effect of estradiol in euhydrated rats treated with dipsogenic stimuli, it is critically important to understand if those results generalize to more physiologically relevant paradigms. The findings in this manuscript provide that critical information and identify avenues for future research aimed at understanding the mechanisms underlying the anti-dipsogenic effect of estradiol.

CRediT authorship contribution statement

Julia A. Howell: Writing – review & editing, Methodology, Data curation, Conceptualization. **Andrea A. Edwards:** Writing – review & editing, Methodology, Data curation. **Jessica Santollo:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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