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Activation of G protein-coupled estrogen receptor 1 (GPER-1) decreases fluid intake in female rats

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Abstract

Estradiol (E2) decreases fluid intake in the female rat and recent studies from our lab demonstrate that the effect is at least in part mediated by membrane-associated estrogen receptors. Because multiple estrogen receptor subtypes can localize to the cell membrane, it is unclear which receptor(s) is generating the anti-dipsogenic effect of E2. The G protein-coupled estrogen receptor 1 (GPER-1) is a particularly interesting possibility because it has been shown to regulate blood pressure; many drinking-regulatory systems play overlapping roles in the control of blood pressure. Accordingly, we tested the hypothesis that activation of GPER-1 is sufficient to decrease fluid intake in female rats. In support of this hypothesis we found that treatment with the selective GPER-1 agonist G1 reduced AngII-stimulated fluid intake in OVX rats. Given the close association between food and fluid intakes in rats, and previous reports suggesting GPER-1 plays a role in energy homeostasis, we tested the hypothesis that the effect of GPER-1 on fluid intake was caused by a more direct effect on food intake. We found, however, that G1-treatment did not influence short-term or overnight food intake in OVX rats. Together these results reveal a novel effect of GPER-1 in the control of drinking behavior and provide an example of the divergence in the controls of fluid and food intakes in female rats.

Keywords

Estrogen; estradiol; estrogen receptor; angiotensin II; GPR30; food intake

Introduction

Estrogens, specifically estradiol (E2), have a profound effect on reducing fluid and food intake in females. These effects of E2 have a delayed onset, suggesting that genomic mechanisms are required for the changes in behavior (Graves et al., 2011; Kisley et al., 1999; Santollo et al., 2013). E2 acts at several receptor subtypes. The classically identified estrogen receptor (ER) α and ER β proteins are nuclear receptors that have direct effects on

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gene expression. More recently, membrane-associated estrogen receptors (mER) have been identified that are capable of generating rapid effects on neuronal physiology (Levin, 2008). Although these rapid effects do not appear to require changes in gene expression, the receptors are capable of altering transcription indirectly by activating intracellular signaling cascades (Micevych and Dominguez, 2009; Micevych and Kelly, 2012).

Recent studies from our lab demonstrated that activation of mER decreases overnight water and food intake in female rats (Santollo et al., 2013), but which specific ER subtype(s) mediate these changes remains an open question. One testable hypothesis is that the effects are mediated by the G protein-coupled estrogen receptor 1 (GPER-1; previously referred to as the orphan receptor GPR30). Activation of GPER-1 by estrogens initiates a number of signaling cascades including activation of mitogen-activated protein (MAP) kinase family members (e.g., extracellular signal-related kinase 1/2; ERK1/2), activation of phosphatidylinositol-3-kinase (PI3K), generation of cAMP, and calcium mobilization (Meyer et al., 2011; Mizukami, 2010). These signaling cascades can produce rapid physiological changes and also can initiate gene transcription, the behavioral consequences of which may take hours or days to become observable.

Proper control of fluid intake is an essential component of blood pressure regulation (Daniels and Fluharty, 2009). In this sense, the known effects of GPER-1 on the maintenance of cardiovascular homeostasis (De Francesco et al., 2013; Lindsey et al., 2009; Martensson et al., 2009) make it tempting to speculate that GPER-1 affects fluid intake, perhaps more proximally than any potential effects on food intake. For example, GPER-1 activation decreases mean arterial blood pressure in hypertensive OVX mRen2.Lewis rats (Lindsey et al., 2009). Whether or not any blood pressure effects of GPER-1 are accompanied by or result from altered fluid intake remains an open question, but GPER-1 mRNA has been localized to nuclei involved in controlling fluid intake (Brailoiu et al., 2007). We therefore hypothesized that GPER-1 is at least in part responsible for fluid intake control in female rats. As an initial step toward testing this hypothesis, we evaluated the effect of all mER subtypes in acutely stimulated water intake and then tested the hypothesis that selective activation of GPER-1 is sufficient to decrease water intake. We also extended these studies to investigate the role of GPER-1 on saline intake because salt, in addition to water, is necessary for proper fluid balance, and estrogens exert an inhibitory effect on intake on both saline and water (Santollo and Daniels, 2015). In addition, we tested for delayed and rapid effects on fluid intake after GPER-1 activation to provide clues about the relevant intracellular actions of GPER-1.

Fluid and food intakes are often closely associated in rats, suggesting that any effects on fluid intake might generalize to food intake. In this respect, the paucity of relevant studies limits our understanding of any potential feeding effects of GPER-1 activation. There is a small number of relevant reports, however, the conclusions that can be drawn from these reports are clouded by discrepant results. For example, studies using GPER-1 knockout mice either found no relevant phenotype resulting from the knockout, or found changes in body weight and visceral adiposity (Liu et al., 2009; Wang et al., 2008). In addition to it being unclear if there is, in fact, an effect of GPER-1 on energy homeostasis, whether or not food

intake underlies this potential effect remains underexplored, especially in rats. We therefore tested the hypothesis that GPER-1 activation altered food intake.

Materials and methods

Animals and Housing

Female Long Evans rats (Harlan Laboratories, Indianapolis, IN) weighing 175–200g upon arrival into the facility were used in all of the studies described. Rats were singly housed in hanging wire-mesh stainless steel cages with *ad libitum* access to food (Teklad 2018; Harlan Laboratories) and tap water unless otherwise noted. Rats in double-bottle intake tests (Experiments 2A and 3A) had continuous access to an additional bottle containing a 1.5% saline solution. All testing occurred in the rat's home cages. The temperature- and humidity-controlled colony room was maintained on a 12:12 h light-dark cycle (lights on at 0700 h). All experimental protocols were approved by the Animal Care and Use Committee at the University of Buffalo, and the handling care of the animals was in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals*.

Surgery

Rats were anesthetized with an injection of a mixture of ketamine (i.m.; 80 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (i.m.; 4.6 mg/kg; Akron Inc., Decatur, IL) and underwent bilateral ovariectomy surgery (OVX) as previously described (Santollo et al., 2013). In the same surgery, a chronic cannula was implanted (coordinates: 0.9 mm posterior and 1.4 mm lateral to bregma and 2.8 mm ventral to the surface of the skull) to allow delivery of substances to the right lateral ventricle as described previously (Santollo et al., 2013; Santollo et al., 2014) (rats in Experiment 5A were OVX, but not implanted with cannula). After surgery, all rats received a single injection of carprofen (sc, 5 mg/kg; Pfizer Animal Health, New York, NY) as a perioperative analgesic. One week later, accurate cannula placement was verified by measuring the drinking response to an injection of 10 ng angiotensin II (AngII, Sigma-Aldrich, St. Louis, MO). Only rats that drank at least 5 ml in 30 min after AngII-treatment were included in the study.

Drugs

17 β -Estradiol 17-Hemisuccinate: BSA (E2-BSA; Batch #R282; Steraloids Inc., Newport, RI) was used in Experiments 1 and 4. To minimize free E2 in the solutions, all E2-BSA solutions were passed through a 3 kDa cutoff filter (Millipore, Billerica, MA) and spun at 16,110 g for 10 min. The filters were washed with 5% DMSO, spun for another 10 min at 16,110 g and washed again with 5% DMSO before being spun for 30 min at 16,110 g. The retentate was resuspended in 5% DMSO immediately before use. After filtration, the resuspended retentate was either diluted 1:10 to produce the initial dose (approximately 500 nM; referred to as E2-BSA) or not diluted to produce a higher dose (approximately 5 μ M; referred to as HE2-BSA). Water soluble β -estradiol (wE2, Sigma-Aldrich, St. Louis, MO) or its control 2-Hydroxypropyl- β -cyclodextrin (control; Sigma-Aldrich, St. Louis, MO) were dissolved in sterile TBS and used in Experiment 4. This form of E2 was synthesized with the addition of a carrier molecule, 2-Hydroxypropyl- β -cyclodextrin, which accounted for 95.31% of the drug dry weight in order to make the E2 water soluble. The amount of drug

was adjusted so that 20 µg of E2 was present in the injected solution diluted in sterile TBS. These doses were chosen based on pilot work in our laboratory. (±)-1-[(3aR*,4S*,9bS*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone (G1, Azano biotech, Albuquerque, NM) dissolved in 100% sterile DMSO was used in Experiments 2, 3, and 5. AngII dissolved in sterile TBS was used in Experiments 1–4.

Fluid intake measures—Total intake was calculated by weighing the water bottles before and after each test period. Licking behavior was measured using a contact lickometer (designed and constructed by the Psychology Electronics Shop, University of Pennsylvania, Philadelphia, PA) that recorded individual licks to allow for analysis of drinking microstructure. The lickometer interfaced with a computer using an integrated USB digital I/O device (National Instruments, Austin, TX). Home cages were affixed with an electrically isolated metal plate with a 3.175-mm-wide opening, through which the rat needed to lick to reach the drinking spout, minimizing the possibility of nontongue contact with the spout.

Experiment 1: Does activation of mER influence AngII-stimulated water intake?

We previously demonstrated that activation of mER decreases overnight water and food intake (Santollo et al., 2013). To determine that the decrease in water intake was a direct effect of mER and not secondary to the decrease in food intake, we measured the effect of mER on water intake stimulated by injection of AngII because the drinking that occurs after AngII is not accompanied by increased food intake. Approximately 2 h into the light phase rats (n=11) were given intracerebroventricular (icv) injections of either 1 µl E2-BSA or vehicle (5% DMSO). Eight hours later, rats received a second 1-µl icv injection of 100 ng AngII. Thirty-minute water intake was measured by weighing the water bottles at the start and finish of the testing period. Food was available throughout the experiment. The timing of these injections was based on pilot studies demonstrating that icv wE2 decreases fluid intake stimulated 8 h later by AngII. One week later the experiment was repeated and rats received the counterbalanced treatment to achieve a within subjects design.

Experiment 2: Does activation of GPER-1 influence AngII-stimulated fluid intake?

Next we tested if the decrease in water intake after mER activation is mediated, at least in part, by the GPER-1 subtype. Approximately 2 h after lights on, rats (n = 11) were treated icv with 1 µl of 0, 25, or 50 µg of the GPER-1 agonist G1, using a counterbalanced within subject design. Eight hours later, rats received a second 1-µl icv injection of 100 ng AngII. Rats had access to tap water and 1.5% saline (Experiment 2A) or a single bottle of tap water (Experiment 2B). All rats were run in both experiments with half of the subjects placed in the single-bottle test first and the other half in the two-bottle experiment first. Thirty-minute fluid intake was measured by weighing the water bottles at the start and finish of the testing period. Food was available throughout the experiment. These doses of G1 were chosen based on published reports using G1 within this range (Lebesgue et al., 2009; Lu et al., 2009). Testing occurred every five to seven days.

Experiment 3: Does activation of GPER-1 rapidly influence AngII-stimulated fluid intake?

As a mER, GPER-1 can have rapid effects on neuronal physiology. We, therefore, tested whether activation of GPER-1 could rapidly influence fluid intake. To this end, during the mid-light phase, rats (Experiment 3A, $n = 10$; Experiment 3B, $n = 8$) were treated icv with 1 μ l of 0, 25, or 50 μ g of G1 using a counterbalanced within subject design. Immediately after the injection of G1, rats were given a second 1- μ l icv injection of 100 ng AngII. Rats had access to tap water and 1.5% saline (Experiment 3A) or a single bottle of tap water (Experiment 3B). Thirty-minute fluid intake was measured by weighing the water bottles at the start and finish of the testing period. Food was available throughout the experiment. Testing occurred every five to seven days.

Experiment 4: Does activation of mER or ER rapidly influence AngII-stimulated water intake?

To further explore the role of rapid mER activation on fluid intake, we tested whether selective activation of mER or activation of all ER subtypes rapidly influenced AngII-stimulated water intake. During the mid-light phase, rats were treated icv with either 1 μ l E2-BSA or vehicle (Experiment 4A, $n = 7$), 1 μ l HE2-BSA or vehicle (Experiment 4B, $n = 7$) or 1 μ l 20 μ g wE2 or vehicle (Experiment 4C, $n = 9$) using a counterbalanced within subject design. Rats then were immediately treated with a second 1- μ l icv injection of 100 ng AngII. Thirty-minute water intake was measured by calculating the difference in the weight of the water bottles at the start and the finish of the testing period. Food was available throughout the experiment. Testing was repeated one week later with rats receiving the opposite treatment to achieve a balanced within-subject design.

Experiment 5: Does activation of GPER-1 influence food intake?

Some reports suggest that GPER-1 is involved in regulating body weight (Wang et al., 2008). Because food intake contributes to the maintenance of body weight we tested whether activation of GPER-1 influenced food intake. Thirty minutes before lights off, rats ($n = 7$) were treated s.c. with 0, 12.5, 25, 50 or 100 μ g G1 using a counter-balanced within subject design. The s.c. route of G1 administration was used here to avoid cannulation procedures that were not otherwise needed for the experiment. The subsequent 24 h food intake was measured (Experiment 5A). Next, to determine whether activation of GPER-1 could rapidly influence food intake, rats ($n = 10$) were food deprived overnight (Experiment 5B) and then given brain injections of 1 μ l of 0, 25, or 50 μ g G1. Food was returned and intake was measured 5, 10, 15, 30, 60, 120 min and 24 h later. In addition, daily body weight was measured throughout both experiments. A counter-balanced repeated measures design was used with testing occurring every seven days.

Data Analysis

Data are presented as means $\pm$ SEM throughout. Fluid intakes, 24 h change in body weight after G1 treatment and 24 h food intakes were analyzed by t-tests or one way ANOVAs. Drinking microstructure analysis (burst size and burst number) was processed in a MATLAB (MathWorks, Natick, MA) software environment before being exported to Excel (Microsoft, Redmond, WA) for final analysis. A burst was defined as at least two licks with

an interlick interval (ILI) of no more than 1 sec. Burst size was defined as the average number of licks within a burst. Drinking microstructure was analyzed with a one way ANOVA. Stimulated non-cumulative food intake was analyzed by a two factor ANOVA (drug X time) with food intake at the 30, 60 and 120 min time periods being normalized to intake/5 min to achieve a consistent bin size. Newman Keul's post hoc test was used to determine group differences following significant ANOVA results. Cohen's d effect size was calculated using the Campbell Collaboration Effect Size Calculator and η^2 was calculated as the $SS_{\text{between}}/SS_{\text{total}}$.

Results

Experiment 1: Does activation of mER influence AngII-stimulated water intake?

Rats given E2-BSA drank less water in response to AngII than did rats given vehicle ($t_{10} = 2.48$, $p < 0.05$, $d = .86$; Fig 1).

Experiment 2: Does activation of GPER-1 influence AngII-stimulated fluid intake?

After G1 treatment rats drank less saline in response to AngII than did rats given a vehicle treatment ($F_{2,18} = 8.96$, $p < 0.01$, $\eta^2 = 0.50$; Fig 2A). Both doses of G1 significantly decreased 30 min saline intake ($p < 0.05$). There was, however, no effect of G1 treatment on AngII-stimulated water intake ($F_{2,18} = 1.84$, $p = \text{n.s.}$, $\eta^2 = 0.17$; Fig 2B). To rule out any possible confounding effects that saline intake may have on water intake, this experiment was repeated but with access restricted to a single bottle of water. Again, G1 treatment did not affect water intake after AngII ($F_{2,18} = 0.37$, $p = \text{n.s.}$, $\eta^2 = 0.04$; Fig 3).

To further investigate the nature of the inhibitory effect on saline intake after G1-treatment, burst analysis was performed on the licking patterns during the 30 min test period. After G1-treatment, the number of bursts was significantly less than what was observed after vehicle-treatment ($F_{2,18} = 6.52$, $p < 0.01$, $\eta^2 = 0.42$; Table 1). The number of licks/per burst was not influenced by either dose of G1 ($F_{2,18} = 1.35$, $p < \text{n.s.}$, $\eta^2 = 0.13$).

Experiment 3: Does activation of GPER-1 rapidly influence AngII-stimulated fluid intake?

Experiment 2 used injections of G1 8 h before rats received AngII. To test for more rapid effects of G1, we repeated the experiment, but instead gave the G1 immediately before AngII. In this experiment, AngII-stimulated saline intake was not affected by G1 treatment ($F_{2,18} = 0.01$, $p = \text{n.s.}$, $\eta^2 = 0.001$; Fig 4A). Similarly, there was no effect of G1 on water intake ($F_{2,18} = 1.88$, $p = \text{n.s.}$, $\eta^2 = 0.18$; Fig 4B). Again, to rule out any possible confounding effects that saline intake may have on water intake, we repeated the experiment, but rats were only given water to drink. In this experiment, rats given G1 drank less water than did rats given vehicle ($F_{2,14} = 8.16$, $p < 0.01$; $\eta^2 = 0.54$; Fig 5). Both doses of G1 significantly decreased water intake ($p < 0.05$).

Again, to further investigate the nature of the inhibitory effect on water intake after G1-treatment, burst analysis was performed on the licking patterns during the 30 min test period. ANOVA detected a main effect of G1 treatment on the number of bursts ($F_{2,14} = 4.66$, $p < 0.05$, $\eta^2 = 0.40$; Table 2) and posthoc tests showed that this was driven only by the

larger dose of G1, which was associated with fewer bursts when compared with 25 μ g-treatment. The number of licks/per burst was not influenced by either dose of G1 ($F_{2,14} = 2.65$, $p < \text{n.s.}$, $\eta^2 = 0.27$).

Experiment 4: Does activation of mER or ER rapidly influence AngII-stimulated water intake?

Rats given E2-BSA ($t_6 = 0.49$, $p = \text{n.s.}$, $d = 0.25$; Fig 6B) or a higher dose of E2-BSA ($t_6 = 0.49$, $p = \text{n.s.}$, $d = 0.44$; Fig 6B) drank the same amount of water in response to AngII as did rats given vehicle. Rats given wE2 immediately before AngII also showed no change in water intake compared to vehicle treatment ($t_6 = 0.31$, $p = \text{n.s.}$, $d = 0.18$; Fig 6C).

Experiment 5: Does activation of GPER-1 influence food intake?

Overnight food intake was not affected by treatment with any dose of G1 ($F_{5,40} = 1.66$, $p = \text{n.s.}$, $\eta^2 = 0.17$; Fig 7). There was no difference in the change in body weight after G1-treatment ($F_{5,40} = 0.68$, $p = \text{n.s.}$, $\eta^2 = 0.08$; data not shown). Food intake stimulated by overnight food deprivation was also not affected by G1-treatment ($F_{2,18} = 0.74$, $p = \text{n.s.}$; $\eta^2 = 0.004$; Fig 8A), nor was an interaction between G1 and time detected ($F_{10,90} = 0.52$, $p = \text{n.s.}$; $\eta^2 = 0.02$; Fig 8A). A main effect of time was detected ($F_{5,45} = 10.11$, $p < 0.01$; $\eta^2 = 0.32$), with intake decreasing as time increased ($p < 0.05$). In addition, there was no effect of G1 on noncumulative 24 h intake ($F_{2,18} = 0.11$, $p < \text{n.s.}$, $\eta^2 = 0.02$; Fig 8B) or on cumulative 24 h intake ($F_{2,18} = 184$, $p < \text{n.s.}$, $\eta^2 = 0.17$; data not shown). Furthermore, there was no effect of G1-treatment on changes in 24 h body weight ($F_{2,18} = 0.42$, $p < \text{n.s.}$, $\eta^2 = 0.04$; data not shown).

Discussion

Decreased fluid intake by E2 has been well documented since the 1970s, but the relevant receptor populations underlying these changes remains unknown (Danielsen and Buggy, 1980; Findlay et al., 1979; Tarttelin and Gorski, 1971). The experiments described here provide the novel finding that non-selective activation of mER is sufficient to decrease acutely stimulated water intake in female rats. The observed effect was present when the treatment was given 8 h before the drinking stimulus, suggesting that genomic changes are required. Our results also show that activation of GPER-1, a mER subtype that is capable of influencing gene expression, is sufficient to decrease AngII-stimulated saline intake, but not water intake, with the same time course as non-selective activation of mER subtypes. Although these data demonstrate that GPER-1 is involved in mediating E2's anti-natriorexigenic effect, there was no change in water intake after GPER-1 activation suggesting that it is not the receptor responsible for the anti-dipsogenic effect observed after non selective mER activation. Surprisingly, when GPER-1 was targeted selectively, we also found rapid effects on water intake that did not extend to saline intake. To further explore the role of GPER-1 in the control of ingestive behavior in female rats, we tested whether activation of GPER-1 influences food intake. These experiments did not reveal any change in overnight or stimulated food intake after GPER-1 activation. Together these results demonstrate a role for GPER-1 in mediating at least some of the effects of E2 on fluid

intake, and show a divergence in the control of water and saline intakes and fluid and food intakes in the female rat.

Our finding that stimulated water intake was decreased after non-selective activation of mER replicates and extends our previous report showing that activation of mER decreases overnight water intake (Santollo et al., 2013). Whereas overnight food intake was also decreased in our previous study, allowing for the possibility that the decreased fluid intake was secondary to a hypophagic effect of the treatment, the present study stimulated drinking in a way that is accompanied by little or no food intake. We, therefore, conclude that the change in water intake observed here, and likely in our initial experiment, was not secondary to a change in food intake. Accordingly, these results demonstrate a selective effect of mER activation on the control of water intake in females.

Identifying which mER subtype(s) mediate the change in fluid intake was our next goal. The 8-h timeframe of action suggests an involvement of genomic mechanisms and could provide some insight into the properties of the receptor(s) involved. Given what is known about the classical transcriptional activity of ER subtypes, the long timeframe might suggest the involvement of a nuclear receptor. This perspective ignores, however, a more recent understanding of E2 actions that expand the classical direct effects of ER α or ER β on gene expression to include indirect transcriptional effects via activation of second messenger systems initiated by E2 binding to GPER-1, ERX, and Gq-mER. This view has been further expanded by the recognition that ER α and ER β also can localize to the plasma membrane and influence gene expression indirectly by activating second messenger systems (Micevych and Dominguez, 2009; Micevych and Kelly, 2012). Although many of these receptors potentially control fluid intake, we focused on GPER-1 because of its known involvement in the maintenance of blood pressure (Meyer et al., 2011), and the critical coordination of fluid intake and cardiovascular homeostasis (Daniels and Fluharty, 2009).

In order to identify which receptor(s) are involved in mediating the anti-dipsogenic effect after non-selective mER activation we tested and found support for the hypothesis that GPER-1 is involved in controlling the fluid intake effects. Furthermore, we examined changes in saline intake because both water and saline intake are necessary for proper fluid balance and intake of both fluids is decreased by E2. Activation of GPER-1 decreased AngII-stimulated saline intake, but not water intake, within the same time interval that mER activation decreased AngII-stimulated water intake. This suggests that GPER-1 is not the receptor involved in mediating the anti-dipsogenic effect of non-selective mER activation (Exp. 1); however, it is involved in mediating the well documented anti-natriorexigenic effect of E2. Furthermore, the decrease in saline intake was mediated by a selective reduction in the number of licking bursts, which, based on previous research, suggests a change in post-ingestive feedback (Davis, 1989; Davis et al., 1999). Indeed, we demonstrated previously that activation of mER decreases water intake through a selective reduction in burst number (Santollo et al., 2013). Therefore, the current study provides additional support for the role of membrane-associated estrogen receptors in mediating post-ingestive feedback signals.

A role of GPER-1 that is limited to saline intake may have been predicted based on the expression pattern of the receptor. GPER-1 is expressed in both the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, two important structures in fluid homeostasis (Brailoiu et al., 2007; Sakamoto et al., 2007). Although these regions are likely involved in both saline and water balance, GPER-1 is highly co-localized with oxytocin, a peptide more selectively involved in the control of saline intake. Specifically, 95% of oxytocin neurons in these brain regions co-express GPER-1 (Sakamoto et al., 2007), and oxytocin plays an important role in the inhibition of saline intake (Blackburn et al., 1992; Blackburn et al., 1993). Accordingly, determining how GPER-1 acts within these brain areas to reduce saline intake is an important next step. One possibility is that GPER-1 activation reduces the expression of the angiotensin type 1 receptor (AT1R) in these brain areas. The time required for this may underlie the difference in the effect observed when G1 was given immediately before or 8 h before the stimulus to drink saline. Consistent with this, 24-hr activation of GPER-1 in both liver epithelial cells and in aortic tissue down-regulates AT1R mRNA (Koganti et al., 2014). Testing this hypothesis seems to be a logical next step to improve our understanding of the mechanism accounting for the present behavioral observations.

Our finding that GPER-1 is involved in controlling fluid intake, which is a critical component for blood pressure regulation, supports and extends recent studies that demonstrate a role for GPER-1 in controlling cardiovascular function in mice, rats, and humans. The expression of GPER-1 in tissues associated with the cardiovascular system along with an increase in expression in hearts of spontaneously hypertensive rats suggests a role of GPER-1 in controlling cardiovascular tone (Bopassa et al., 2010; Broughton et al., 2010; De Francesco et al., 2013; Ding et al., 2009; Isensee et al., 2009). Indeed, female GPER-1 knockout mice are hypertensive (Martensson et al., 2009) and the hypotensive effect of pharmacological GPER-1 activation is lost in male and female GPER-1 knockout mice (Haas et al., 2009). *In vivo* pharmacological studies demonstrate that normotensive male rats can be made hypotensive by activation of GPER-1 (Haas et al., 2009) and *in vitro* pharmacological studies show that GPER-1 activation dilates arterial blood vessels in rats, mice, and humans (Haas et al., 2009), and reduces contractility in isolated and Langendorff-perfused male rat hearts (De Francesco et al., 2013). The mRen2.Lewis rat, a hypertensive strain that has exaggerated hypertension after estrogen depletion by OVX, has been a recent model for investigating the role of GPER-1 in cardiovascular health. One such study found that two weeks of G1 treatment decreases blood pressure in OVX mRen2.Lewis rats (Lindsey et al., 2009). Additionally, G1-treatment reduces AngII-induced constriction in isolated aortic rings from OVX mRen2.Lewis rats (Lindsey et al., 2009) and induces vasodilation in heart vessels from OVX mRen2.Lewis rats that is similar in magnitude as that observed after E2-treatment (Lindsey et al., 2011). Given the critical role fluid intake plays in maintaining cardiovascular homeostasis, our data demonstrating a role of GPER-1 in fluid intake further suggest its role in the maintenance of cardiovascular health.

To further explore a role for GPER-1 in the control of fluid intake we tested the relevant hypothesis that rapid actions of GPER-1 affect AngII-stimulated fluid intake. A rapid effect is a possibility because, as discussed above, GPER-1 is a membrane-associated receptor and

therefore capable of rapidly altering neuronal activity and in turn behavior. Our experiments found partial support for this hypothesis by showing rapid effects on water intake; however, saline intake was unaffected by the treatment. Saline intake was, however, suppressed by GPER-1 activation 8 h before the saline intake was stimulated. This suggests a transcriptional requirement for the effects on saline intake that is not critical for the decrease in water intake. As described above, the effects on saline intake may require changes (e.g., AT1R or oxytocin) that are not needed for the effects on water intake. Nevertheless, the rapid influence on water intake was surprising because past reports suggest that inhibitory effects of E2 on fluid intake are genomically mediated (Graves et al., 2011). The result is especially interesting in light of the results of other present experiments. Specifically, we did not find any rapid intake effects caused by injection of E2-BSA or E2. Because these treatments should activate all mER or all ER, respectively, it suggests that there is something different about selective activation of GPER-1 in the absence of a contribution from other receptor subtypes. It is possible that because we used a repeated measures design the rapid decrease in water intake could be the result of a delayed genomic effect from the previous test day. We think this interpretation is unlikely, however, because testing occurred no more than once every five days. Drawing any firm conclusions regarding the nature of this difference, however, requires further experimentation.

The observed effects of GPER-1 activation on fluid intake suggest that delayed and rapid effects underlie the changes in behavior. Although delayed genomic changes can be mediated by both nuclear and membrane-associated receptors, rapid changes are most often ascribed to receptors that are associated with the plasma membrane. It is important to note, however, that GPER-1 has not only been shown to associate with the plasma membrane (Sakamoto et al., 2007), but others have demonstrated GPER-1 associated with the endoplasmic reticulum and Golgi apparatus (Funakoshi et al., 2006; Sakamoto et al., 2007). Accordingly, we cannot be certain that the presently observed rapid changes in water intake resulted from plasma membrane-associated GPER-1. Nevertheless, the uncertainty of the cellular localization of GPER-1 should not diminish the main finding that GPER-1 activation did indeed influence fluid intake.

In addition to the main fluid intake focus of our studies we tested the hypothesis that GPER-1 activation decreases food intake, thereby causing secondary effects on fluid intake. We found no support for this hypothesis in experiments using overnight intake or intake after food-deprivation. Experiments using overnight intake or acute intake after deprivation tested for longer onset genomic effects, or acute-onset rapid effects, respectively, and found no effect of either on food intake. To our knowledge this is the first test of GPER-1 activation and food intake in female rats, although the results are consistent with a previous study that reported no effect of GPER-1 activation on body weight in mRen2.Lewis rats (Lindsey et al., 2009). Furthermore, we found no effect of GPER-1 activation on body weight. Comparing the results of these studies with those conducted using mouse models is problematic, however, and the results of studies using mice tend to be inconsistent. Specifically, an obese phenotype (Wang et al., 2008) and a lack of an obese phenotype (Liu et al., 2009) have been reported using a knockout model developed by Wang et al. (Wang et al., 2008). Other GPER-1 knockout models lack an obese phenotype (Isensee et al., 2009; Otto et al., 2009) or have an abnormally lean phenotype (Martensson et al., 2009). Another

study reported that reduction in fat mass after E2-treatment is equally effective in wildtype and GPER-1 knockout mice (Windahl et al., 2009), but this is in opposition to findings from a different report demonstrating that the inhibitory effect of E2 on body weight is absent in female GPER-1 knockout mice (Davis et al., 2014). Although most of these reports do not include direct measures of food intake, Davis et al. (Davis et al., 2014) found no change in average daily food intake but did find a disruption of the estrous cycle-associated changes in food intake in the female GPER-1 knockout model generated by Wang et al. (Wang et al., 2008). In addition, the anorexigenic effect of leptin and CCK were lost in the GPER-1 deficient female but not male mice (Davis et al., 2014). These data suggest a role of GPER-1 in mediating E2's anorexigenic effects in female mice, counter to what we observed presently in female rats. More recently, however, Ervin et al. (2015) found that G1 did not affect total food intake in a study of social transmission of food preferences in female mice. This finding is consistent with the results of our present experiments using rats. Accordingly, additional research is needed to reconcile the conflicting reports from the knockout models and to understand any species differences that may be apparent in the literature.

In conclusion, these findings provide further support that membrane-associated ER subtypes play an important role in the fluid intake effects of E2 and highlight a role for GPER-1. The findings strongly suggest that GPER-1 plays a key role in the anti-natriorexigenic effect of E2 in female rats. Understanding the mER subtype(s) involved in mediating the anti-dipsogenic effect of E2 and elucidating the timing dependent differences on fluid intake are important areas for future research. Determining a mechanism of action for the effects observed here may ultimately help further our understandings of the biology that underlies cardiovascular health in females.

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Highlights

Activation of membrane-associated estrogen receptors selectively reduces drinking.

Activation of GPER-1 decreases saline intake.

Activation of GPER-1 rapidly decreases water intake.

Activation of GPER-1 decreased fluid intake by decreasing post-ingestive signals.

GPER-1 activation has no influence on short-term or overnight food intake.

Figure 1. Non-selective activation of mER decreased water intake. Treatment with E2-BSA reduced 30 min AngII-stimulated water intake. *Less than Vehicle, $p < 0.05$.

Figure 2.

Activation of GPER-1 decreased fluid intake. AngII-stimulated saline intake was decreased after treatment with 25 and 50 µg G1 (A); however, water intake was unchanged (B). *Less than Vehicle, $p < 0.05$.

Figure 3.

Activation of GPER-1 had no effect on AngII stimulated water intake. Neither dose of G1 influenced 30 min AngII-stimulated water intake.

Figure 4.

Activation of GPER-1 had no rapid effect on 30 min AngII-stimulated fluid intake in a two-bottle test. Neither AngII-stimulated saline (A) or water (B) intake was affected by any dose of G1 treatment.

Figure 5.

Activation of GPER-1 had a rapid effect on AngII-stimulated water intake in a single bottle test. Both 25 and 50 μg of G1 rapidly decreased 30 min AngII-stimulated water intake.

*Less than Vehicle, $p < 0.05$.

Figure 6.

ER activation did not have a rapid influence on AngII-stimulated water intake. Neither E2-BSA (A), a higher dose of E2-BSA (HE2-BSA; B), nor estradiol (C) had any effect on 30 min Ang-II stimulated water intake.

Figure 7.

Activation of GPER-1 did not influence food intake. Unstimulated 24 h food intake was not influenced by treatment with any dose of G1.

Figure 8.

Activation of GPER-1 did not have a rapid effect on stimulated food intake. Food intake stimulated by overnight food deprivation was not influenced by any dose of G1 (A). Data are shown as cumulative intake within each bin, but were analyzed as intake per 5 min to account for the different bin sizes. In addition, noncumulative 24 h food intake was not influenced by any dose of G1 (B).

Table 1

Burst analysis of saline intake after delayed G1-treatment.

Dose (μg)	Burst number ($\pm$ SEM)	Licks/burst (mean $\pm$ SEM)
0	41.7 $\pm$ 6.11	111.19 $\pm$ 19.17
25	23.1 $\pm$ 2.96*	96.52 $\pm$ 15.44
50	29.1 $\pm$ 4.26*	118.93 $\pm$ 13.85

*
p<0.05 compared with 0 μg .

Table 2

Burst analysis of saline intake after rapid G1-treatment.

Dose (μg)	Burst number ($\pm$ SEM)	Licks/burst (mean $\pm$ SEM)
0	43.2 $\pm$ 5.76	79.85 $\pm$ 13.82
25	47.8 $\pm$ 6.31	62.57 $\pm$ 11.17
50	36.0 $\pm$ 3.52 *	73.50 $\pm$ 13.27

*
p<0.05 compared with 25 μg .