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## Oestrogen Induces Rhythmic Expression of the Kisspeptin-1 Receptor 1 GPR54 in Hypothalamic Gonadotrophin-Releasing Hormone (GnRH)-Secreting GT1-7 Cells

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### Abstract

Oestrogen-stimulated preovulatory gonadotrophin surges are temporally regulated in a way which remains not fully understood. Mammalian ovulation requires surges of gonadotrophin-releasing hormone (GnRH), released from specialized neurones in the hypothalamus. Surge regulation is mediated by ovarian oestrogen (E2) feedback- acting as a negative signal until the early afternoon of the pro-oestrus phase, at which point it stimulates robust increases in GnRH release. Multiple lines of evidence suggest a role for the circadian clock in surge generation, but the presence of endogenous oscillators in several neuronal populations throughout the mediobasal hypothalamus complicates elucidating the underlying mechanisms of circadian regulation. In this study, we propose that endogenous oscillators within GnRH neurones are modulated by oestrogen to elicit GnRH surge secretion. One mechanism by which this may occur is through the upregulation of receptors of known stimulators of GnRH, such as kisspeptin's cognate receptor, GPR54. Through analysis of mRNA and protein abundance patterns, we found that high levels of E2 elicit circadian expression profiles of *GPR54 in vitro*, and that disruption of endogenous GnRH oscillators of the clock dampens this effect. Additionally, while kisspeptin administration to GT1-7 cells does not result in surge-level secretion, we observed increased GnRH secretion from GT1-7 cells treated with positive feedback levels of E2. These results in this *in vitro* neuronal model system suggest a possible mechanism whereby receptor expression levels, and thus GnRH sensitivity to kisspeptin, may change dramatically over the pro-oestrus day. In this way, elevated ovarian E2 may increase kisspeptidergic tone while simultaneously increasing GnRH neuronal sensitivity to this neuropeptide for maximal surge release.

### Introduction

The regulation of ovulation in female mammals requires precise temporal orchestration of neuronal cues, as positive feedback levels of ovarian E2 must be integrated with hypothalamic neural signals to elicit preovulatory surges of gonadotrophin-releasing hormone (GnRH) (1). Seen most strikingly in rodents, GnRH and subsequent LH surges are tightly temporally confined to the late afternoon of the pro-oestrus phase, such that pharmacological manipulations delay surges until the same time the following day (2, 3). Additionally, elevated exogenous E2 treatment following ovariectomy elicits LH surges, peaking around the initiation of darkness, for several consecutive days (4). Recently, the RFamide peptide kisspeptin has been shown to play an important role in neuroendocrine

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regulation of reproduction, including pubertal initiation and ovulation. While kisspeptin (also known as metastin) was initially identified for its anti-metastatic properties (5), further investigation implicates kisspeptin as a potent stimulator of GnRH neurones (6–11), making it a crucial component of the hypothalamus-pituitary-gonad axis. Kisspeptin knockout mice, as well as mice harboring a deletion of kisspeptin's cognate receptor, GPR54, are infertile (12, 13), exhibiting a phenotype similar to human idiopathic hypogonadal hypogonadism. Administration with exogenous GnRH can ameliorate this hypogonadism, suggesting that this neuropeptide exerts its effects on GnRH neurones (14). Kisspeptin neurones represent one likely integration site for ovarian E2, since nearly all kisspeptin neurones, both in the arcuate nuclei (ARC) and anteroventral periventricular nuclei (AVPV), express ER $\alpha$  (15). In most mammals, it is estimated that >85% of GnRH neurones in the median eminence (ME) express the kisspeptin receptor GPR54 (16), and 70–90% of GnRH perikarya are in close proximity to kisspeptinergic neuronal terminals (11). This, along with evidence that kisspeptin increases during the LH surge (17), antiserum of kisspeptin can block the LH surge (18), and infusion of novel GPR54 antagonists can inhibit GnRH and LH surge release in rodents and primates (19) lend credence to the model that kisspeptin is an essential mediator of the preovulatory GnRH surge.

Recent evidence additionally suggests that the temporal control of the neuroendocrine regulation of GnRH neurosecretion may involve dynamic changes in kisspeptin expression and release. In this model, kisspeptin-expressing neurones in the AVPV act as a cellular integrator of temporal neural signals originating from the proximally caudal suprachiasmatic nuclei (SCN), and hormonal cues in the form of elevated ovarian oestradiol levels from mature follicles (17). Consistent with this, recent studies have observed E2-dependent increases in vasopressinergic projections from the SCN (20), and that vasopressin can induce c-Fos expression in AVPV kisspeptinergic cells (21). Daily signals, as yet uncharacterized, from the SCN have long been thought to be required for LH surge generation, based on previous ablation studies (22, 23). In addition to a role of afferent signals from the SCN, more recent studies have demonstrated the presence of a peripheral circadian oscillator within GnRH neurones which is capable of functioning independently of the SCN (24–26). The immortalised GnRH-expressing and –secreting cell line, GT1-7, has been shown to possess functional endogenous circadian oscillators (24, 26), which may play a role in the intrinsic secretory mechanism in these neurones. While it remains unknown if endogenous GnRH cell-specific clocks are obligate for normal preovulatory surge generation, previous evidence in circadian clock mutant and knockout mice suggests a role for cellular clocks in the neuroendocrine regulation of normal reproductive function (24, 27–29).

In studies described below, we explored the possibility that E2 may mediate a portion of its surge-generation influence directly at the level of the GnRH neurone by modulating expression patterns of receptors of GnRH-stimulatory factors, namely kisspeptin. Temporal sensitization of GnRH neurones to the effects of kisspeptin could represent one of many redundant mechanisms designed to increase GnRH secretion in a time-gated fashion. Using the GT1-7 cell line as a homogenous neuronal model system in which to directly address these cellular changes, we have determined that exposure to positive feedback levels of oestradiol can induce or unmask transcriptional rhythmicity of *GPR54* expression in these cells, and that protein abundance of GPR54 is also rhythmic under conditions of elevated E2, a phenomenon which appears to be mediated by intracellular ER $\beta$  receptors. Indeed, it is possible that multiple transcripts may exhibit circadian rhythms of expression following exposure to positive feedback levels of E2, such that this steroid hormone could sensitise GnRH neurones to multiple afferent stimuli, or increase native neuronal activity in a temporally-gated manner, which has been very recently explored (4).

## Methods

### Cell culture and treatments

GT1-7 cells were grown in DMEM (Mediatech, Herndon, VA) with 10% fetal bovine serum (Gemini Bio-Products, Sacramento, CA) and 1.0% Pen-Strep (penicillin; 100 U/ml), and streptomycin (0.1 mg/ml; Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in 5% CO<sub>2</sub>/95% O<sub>2</sub>. To serum-synchronise cells, 10% FBS-DMEM media was replaced with a solution of 50% FBS and 50% serum-free (SF) DMEM for 2 hours. 50% serum-containing media was removed, and at this point, plates of GT1-7 cells were incubated with SF-DMEM containing either 100.0 pM 17-β-oestradiol (Sigma, St. Louis, MO) or ethanol (EtOH) vehicle. Separate plates of confluent GT1-7 cells were treated with 200.0 pM ERα agonist PPT (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol, Tocris Bioscience, Ellisville, MO) or 200.0 pM of ERβ agonist DPN (2,3-bis(4-Hydroxyphenyl)-propionitrile, Sigma).

### Derivation of subcloned GT1-7CikΔ19 cell line

Briefly, GT1-7 cells were stably transfected with the pTet-OFF regulator vector (Clontech, Mountain View, CA) using FuGene (Roche Molecular Diagnostics, Pleasanton, CA). After 24 h, the medium was changed and 900 µg/mL G418 (Invitrogen) added to select for transfected cells. G418-resistant cells were clonally selected by fluorescence activated cell sorting and clones were expanded. The mutant *Clock-Δ19* gene was removed from a pcDNA3.1 construct (gift from J. Takahashi) with BamHI (New England BioLabs, Ipswich, MA) and subcloned into doxycycline-controlled vector pTRE2hyg (Clontech) linearized with BamHI. A clone with the properly oriented gene inserted was identified by restriction digest analysis. ClockΔ19pTRE2hyg was subsequently transfected (using PolyJet; SignaGen Ijamsville, MD) into pTet-OFFGT1-7 cells. After 24 h, the medium was changed and 200 µg/mL hygromycin B (Invitrogen) added to select for transfected cells. Hygromycin-resistant cells were expanded and challenged with G418 to reconfirm the presence of pTet-OFF. Double-transfectant ClockΔ19pTre2hyg/pTet-OFFGT1-7 cells (hereafter referred to as GT1-7CikΔ19 cells) were cultured either in the presence or absence of 10 µM doxycycline hyclate (Sigma-Aldrich). Verification of doxycycline-controlled *Clock-Δ19* mRNA expression was verified by real time qPCR.

### RNA isolation and protein extraction

Cells were harvested every 6 hours for a total of 72 hours for initial 17β-E2 vs. EtOH gene expression study (Figure 1), and for 48 hours for all experiments thereafter. Following removal of media, plates were washed with 1X PBS before Trizol (Invitrogen) reagent was added at a concentration of 2 × 10<sup>6</sup> cells per millilitre. RNA was isolated using a previously described protocol (30), followed by further purification using RNEasy mini kits (Qiagen). Protein extraction was performed in RIPA buffer with proteinase inhibitor cocktail added, and quantified using a bicinonchic acid (BCA) assay (Pierce Biochemicals, Rockford, IL).

### cDNA synthesis and real-time quantitative RT-PCR

2.0µg of total RNA from each sample was converted to cDNA using Sprint tubes (Clontech, Hercules, CA). 5.0 µg of cDNA was amplified using 15.0 µL of reverse transcriptase master mix containing SYBR-Green (Bio-rad). Sequence specific primers were used to amplify GPR54 (see supp. Table 1). Real-time qRT-PCR was performed and data analysis was done using Applied Biosystems 7300 RT-PCR system (Applied Biosystems, Foster City, CA). Negative controls were run without template cDNA. Relative expression levels were determined using the 2<sup>-ΔΔCt</sup> method. Primers were designed to specifically amplify *Clock-Δ19*, with the forward primer spanning the junction of exons 18 and 20. Forward primer: 5'

CAA GGC ATG TCA CAG ATG TT 3' and reverse primer: 5' GGA AAG TTG AAC AGA ACC AAA 3'. PCRs were carried out in a StepOnePlus Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA) using Power SYBR master mix and the following thermal cycler profile: 95°C 10 m initial denaturation, followed by 40 cycle of 95°C 15 s and 60.5°C 30 s. Amplicon specificity was verified by melt-curve analysis using the StepOnePlus data analysis software.

### Western blotting

Extracts from GT1-7s were obtained by protein extraction described above. Concentrations were determined using a BCA Assay (Pierce), and 20.0 µg of DNA was loaded into a 10% SDS-Polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane using the iBlot semi-dry transfer system (Invitrogen). Membranes were washed in a buffer containing 10% bovine serum albumin and 0.1% Tween-20 (Sigma), and probed with goat-anti GPR54 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-COXIV (1:1,000) (Cell Signaling Technology, Danvers, MA). Visualization was performed using Li-Cor IRDye 800 rabbit-anti goat and goat-anti rabbit secondary antibodies, and quantified using a Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Nebraska) Quantification of the bands was performed using Odyssey 2.1 software (Li-Cor).

### Radioimmunoassay

Duplicate 100 µL media samples from 10cm plates of GT1-7 cells were incubated for 48h with EL-14 primary antibody directed against GnRH. Radiolabeled <sup>125</sup>I-GnRH was then incubated with samples for an additional 48h. 3.0mL of 100% ethanol was added to samples (including standard curve tubes) and centrifuged at 2500rpm at 4°C for 15min. Pellets were loaded into a gamma-counter (Packard) and counted for 1 min/sample. Intra-assay CV and inter-assay CV were 3.2% and 6.8%, respectively.

### Statistical Analysis

Comparisons of ethanol (EtOH) vs. 17β-oestradiol (E2) treatment groups across time were carried out using a two-way analysis of variance (ANOVA) with repeated measures, followed by Bonferroni's *post-hoc* test. In all analyses, P<0.05 was considered significant, and demarcated as such.

## Results

### **Experiment 1- Effects of positive feedback levels of E2 on transcript and protein abundance rhythms of GPR54 in GT1-7 cells**

An earlier pilot study using expression microarrays revealed that several transcripts in GT1-7 cells exhibited rhythmic expression patterns only following treatment with 17β-oestradiol. Interestingly, we found that *GPR54* expression levels appeared to exhibit significant rhythms following 24 hours of E2 exposure, in comparison to vehicle-treated controls. To confirm these preliminary results, we treated serum-synchronized GT1-7 cells with 100.0 pM 17β-oestradiol or 0.01% ethanol vehicle in serum-free and phenol red-free DMEM, and collected samples in TriZol every 6h for 72h. Relative expression results from real-time qRT-PCR revealed rhythmic increases in *GPR54* mRNA levels only in E2-treated GT1-7 cells (Figure 1A), which exhibited significant peaks (\*, P<0.05 vs. EtOH, n=4) at 24 hrs and again at 48 hours following E2 treatment. A third peak occurred at 72h following a nadir at 66h, but was not statistically different from levels observed in vehicle-treated cells, due to a steady rise in *GPR54* mRNA in those cells over the sampling period. In agreement with this, levels of GPR54 protein were observed to show similar rhythmic peaks following treatment with 100.0 pM 17β-E2, also peaking at 24 (\*, P<0.05 vs. EtOH, n=4) and 48 hrs

following E2 treatment, although the difference at 48h did not reach statistical significance (Figure 1B). Additionally, since several previous studies have suggested that GnRH neurones express only oestrogen receptor beta (ER $\beta$ ), but not ER $\alpha$ , we used specific agonists to explore if the above effect may be mediated via one of the classical genomic oestrogen receptors. In agreement with the observation that ER $\beta$  likely plays a larger role in GnRH neurones, we observed similarly robust rhythms of *GPR54* transcript abundance following treatment with the ER $\beta$ -specific agonist DPN, but not with the ER $\beta$ -specific agonist PPT (Figure 2).

### **Experiment 2- Effects of estradiol treatment on kisspeptin-stimulated GnRH secretion**

Following confirmation that exposure of GT1-7 cells to positive feedback levels of E2 resulted in rhythmic expression patterns of *GPR54*, we wanted to determine if temporal availability of this receptor resulted in commensurate sensitivity of these cells to kisspeptin stimulation, as measured by GnRH secretion into the culture medium. GT1-7 cells, either E2- or EtOH vehicle-treated, were exposed to 10.0 nM kisspeptin-10 for 45 minutes at 6 hr intervals, and GnRH secretion into the media was quantified by RIA. Kisspeptin-10 is comprised of amino acids 45–54 of the mature peptide, and is equally biologically active as the full protein (31). Similar to what was reported previously (32), kisspeptin-10 stimulated a minor increase in GnRH secretion from GT1-7 cells, but only between 30–48 hrs in serum-synchronized cells (Figure 3B, open squares; \*,  $P < 0.05$  vs. EtOH/DMSO). Interestingly, GT1-7 cells exposed to E2 exhibited significant (*a*,  $P < 0.05$  vs. EtOH/KP-10; *b*,  $P < 0.05$  vs. EtOH) increases in GnRH secretion following Kiss-10 stimulation, with peaks in GnRH secretion occurring at 24 and 48 hours following E2 treatment (Figure 3B, filled squares), correlating temporally to elevated *GPR54* abundance levels observed above in Figure 1. Of note, GnRH secretion from cells exposed to E2 and treated with DMSO vehicle instead of kisspeptin-10 was also both significantly elevated and rhythmic (*c*,  $P < 0.05$  vs. EtOH/DMSO), but exhibited slightly earlier secretory peaks at 18 and 42 hours, suggesting that positive feedback levels of E2 can increase GnRH secretion from GT1-7 cells even without stimulation by kisspeptin, confirming earlier observations using both short- and long-term oestradiol exposure (33).

### **Experiment 3- Determining the requirement of endogenous circadian clock activity for rhythmic abundance patterns of GPR54**

As a final step, we used a subcloned cell line derived from the GT1-7 cell line, GT1-7Clk $\Delta$ 19 (Figure 4A), in which expression of the dominant negative *CLOCK- $\Delta$ 19* transcript is under the control of constitutively expressed TetR-VP16 operons, such that the mutant protein, driven by a tet-responsive element (TRE) can be conditionally repressed by exposure to the antibiotic doxycycline (DOX). Using this subcloned line, we can use DOX removal to initiate mutant protein expression, thus resulting in the rapid and robust disruption of endogenous circadian oscillators, an effect observed previously in this cell line using transient transfection (24). We thus used these cells to investigate the necessity of a functional circadian clock within GnRH neurones for the observed rhythmic abundance patterns of *GPR54* following exposure to positive feedback levels of E2. As compared to GT1-7 cells, GT1-7Clk $\Delta$ 19 cells express the mutant *CLOCK- $\Delta$ 19* protein at high levels, which are reduced over time by exposure to DOX (Figure 4B). Surprisingly, levels of *GPR54* mRNA were also dramatically elevated in GT1-7Clk $\Delta$ 19 cells, the levels of which returned to baseline following treatment with DOX (Figure 4C), suggesting that endogenous clock function may normally serve to keep kisspeptin receptor expression in check.

This increased expression was also observed over a longer temporal scale. As seen in Figure 5, GT1-7Clk $\Delta$ 19 cells treated with either vehicle or 100.0 pM 17 $\beta$ -E2 display elevated and constitutive expression of *GPR54* mRNA (Fig 5A) and *GPR54* protein (Fig 5B), suggesting

the requirement of a functional endogenous oscillator for E2-induced rhythmic expression patterns. Of note, GPR54 expression levels were constitutively higher in GT1-7Clk $\Delta$ 19 cells in comparison to GT1-7 cells, *both* in the absence or presence of E2 exposure, suggesting that E2 is unable to modulate GPR54 expression levels in the absence of a functional circadian oscillator. 24h pretreatment of GT1-7Clk $\Delta$ 19 cells with DOX decreased GPR54 mRNA levels in comparison to untreated cells, (Fig 4C and 5A), and 100.0 pM 17 $\beta$ -E2 treatment elicited a rhythm of GPR54 mRNA only in DOX-treated GT1-7Clk  $\Delta$ 19 cells (Fig 5A; filled squares/dashed lines).

## Discussion

Although it is well established that elevated ovarian estradiol levels are required for LH and antecedent GnRH surge generation, it yet remains unclear how this steroid hormone can function as both a negative and positive feedback signal dependent upon concentration. Positive feedback levels of E2 do not correlate temporally with GnRH secretion above a particular threshold; circulating ovarian oestradiol levels are highest on the morning of pro-oestrus when GnRH secretion remains low (34). Noting the temporal confinement of the GnRH surge, it is thus conceivable that suprathreshold levels of oestradiol serve to initiate cellular processes which prime hypothalamic release patterns to elicit GnRH release governed by a circadian signal. While it is likely that synchronizing signals from the SCN and steroid hormone-sensitive AVPV play a role in surge generation, we chose to investigate possible direct effects of oestradiol on GnRH neurones themselves using the *in vitro* GT1-7 cell model. Recent studies have demonstrated that native GnRH neurones undergo alterations in sensitivity and neuronal activation with a circadian pattern only under the influence of positive feedback levels of oestradiol (4) thus we attempted to determine if one way E2 acts to elicit these changes in GnRH neuronal sensitivity is by affecting temporal patterns of gene expression of receptors for factors such as kisspeptin. Using real-time qRT-PCR and western blotting, we have demonstrated that kisspeptin's cognate receptor, GPR54, exhibits circadian rhythms of expression in GT1-7 cells only following an appropriate duration of elevated oestradiol exposure. GPR54 protein abundance also exhibits a similar rhythm, interestingly without a noticeable lag between mRNA and protein, which may however be reflective of the temporal resolution of sampling, such that more frequent samples may reveal a lag of several hours.

Evidence suggests that E2 may sensitise GnRH neurones to multiple afferent signals, such as glutamate and GABA (35), as well as influencing temporal patterns of cation channel expression and abundance (36), such that increasing sensitivity to kisspeptin may represent one of several redundant mechanisms involved to insure GnRH surge generation. This is demonstrated in the current study in Figure 3, in which elevated and rhythmic GnRH secretion was observed from GT1-7 cells treated with E2 even without exogenous kisspeptin-10 stimulation. Our experiments suggest that E2 directly mediates effects on GT1-7 cell sensitivity and can stimulate GnRH secretion *in vitro* from these cells, and that these effects require a functioning intracellular circadian oscillator. It should be noted, however, that these *in vitro* results have yet to be verified in GnRH neurones *in vivo*, and it remains unclear if GnRH-specific circadian oscillators are required for E2-stimulated GnRH surge generation in animal models. A previous study using GT1-7 cells showed that *GPR54* mRNA exhibited low-amplitude circadian rhythms in the absence of E2 (26), while our current study observed more robust rhythms in the presence of E2. The current study does not elucidate how E2 treatment results in the rhythmic expression of this receptor; however, observation of differential transcript rhythms in the presence of steroid hormones is not unprecedented. Although still incompletely characterized, there is growing evidence that nuclear receptors can interact with core clock machinery (37), including recent evidence that glucocorticoids may act to repress rhythms of core clock and clock-controlled gene

expression in both the brain and periphery (38, 39). Further studies will be required to determine more specifically how steroid hormones acting via either nuclear or membrane receptors might be interacting with endogenous oscillators.

Our results are also consistent with previous work using GT1-7 cells to investigate *GPR54* expression. Specifically, another group noted that E2 stimulated *GPR54* mRNA levels in GT1-7 cells at 24 hours, but did not explore transcript rhythms (40). Interestingly, the GT1-7C1kΔ19 cells, which harbour a disrupted circadian clock, exhibited constitutively elevated GPR54 levels, suggesting that in addition to an increase in GnRH neuronal sensitivity to kisspeptin on proestrus afternoon, the quiescence in GnRH secretion on the morning of pro-oestrus may also be mediated by an endogenous oscillator, keeping GnRH neuronal sensitivity low until the appropriate temporal window. Interestingly, preliminary results suggest that this observed elevation in GPR54 mRNA in GT1-7C1kΔ19 cells does not translate into increases in kisspeptin-stimulated GnRH secretion. Indeed, as observed in Figure 3, kisspeptin only stimulated GT1-7 cells at certain times, an effect that appears to have been potentiated by E2. Since GnRH neurones *in vivo* localize GPR54 to axonal terminals in the ME as well as in perikarya, it remains a possibility that GPR54 in GT1-7 cells are sub-functional due to a lack of this typical morphological configuration *in vitro*, resulting in diminished GnRH secretion following kisspeptin treatment. Another possibility underlying observed differences in kisspeptin's ability to stimulate GnRH secretion *in vivo* vs. *in vitro* could be ascribed to the neurochemical context that is present *in vivo* only, supported by studies demonstrating modulation of GABA and glutamate release on to GnRH neurones *in vivo* (35).

In a very recent article, Robertson et al. demonstrated that *kisspeptin* expression levels in the AVPV increased concomitantly with the LH surge in E2-primed, ovariectomized (OVX) mice, and that the largest percentage of kisspeptin neurones expressing *c-fos* occurred at the same time, around circadian time (CT) 11-12 (17). This increase in hypothalamic *kisspeptin* expression and c-Fos co-localization appears to occur with a circadian period, but only under conditions of positive feedback levels of E2. Interestingly, a *kisspeptin* expression rhythm was not observed in unprimed OVX females, suggesting that E2 is required for inducing increases in *kisspeptin* expression. The necessity of E2 for circadian *kisspeptin* production further supports the presence of steroid-induced circadian rhythms in the hypothalamus. However, in line with the results presented above, a very recent study demonstrates that exogenously applied kisspeptin can only stimulate *c-fos* expression in GnRH neurones *in vivo* dependent on oestradiol exposure and time of day (21), further implicating changes in GnRH neuronal sensitivity gated by steroid milieu interacting with endogenous oscillators. There may also exist redundant mechanisms to ensure temporal control of GnRH surge generation, as earlier studies demonstrated that icv vasopressin injection can induce LH surges in both wild-type and *Clock/Clock* mutant mice, suggesting that increases in SCN-derived neuropeptides may be able to circumvent the absence of an endogenous GnRH oscillator. Interestingly, antagonism of the vasopressin receptor AVPR1A in wild-type mice was unable to prevent surges, raising the possibility that endogenously-timed kisspeptin release and reception may be sufficient for GnRH surge release in the absence of vasopressin signalling (41).

Additionally, our results suggest that this direct effect of E2 on kisspeptin receptor expression levels in GnRH neurones may be mediated by ERβ, the E2 receptor isoform which has been shown to be expressed in these cells (42, 43). ERβ activation in GnRH neurones is likely a functionally redundant mechanism to ensure optimal surge amplitude, however, since mice lacking ERα display a more severe reproductive phenotype than ERβ knockout mice (43). We can also not exclude the possibility that E2 and DPN could activate

incompletely-characterized membrane-associated E2 receptors, which exert their effects via non-classical signalling mechanisms (44).

Future studies will investigate the signalling pathways by which E2 exerts its effects within GnRH neurones, and will explore transcriptional mechanisms underlying how E2 can alter patterns of transcriptional rhythmicity at the level of gene promoters, perhaps by the interaction of ERs with core circadian clock proteins, which has been observed to occur in cancer cell types (45). In addition to demonstrating an additional level of E2 modulation of neuroendocrine control of reproductions, our results suggest a novel form of transcriptional regulation, by which steroid hormones may alter the temporal nature of gene expression via interaction with endogenous cellular oscillators.

## Abbreviations

<b>GnRH</b>	gonadotrophin-releasing hormone
<b>OVX</b>	ovariectomized
<b>E2</b>	oestrogen
<b>LH</b>	luteinising hormone
<b>ER</b>	oestrogen receptor
<b>SCN</b>	suprachiasmatic nuclei
<b>ARC</b>	arcuate nuclei
<b>AVPV</b>	antero-ventral periventricular nuclei
<b>MBH</b>	medio-basal hypothalamus
<b>Kiss-1</b>	kisspeptin-1
<b>icv</b>	intracerebroventricular

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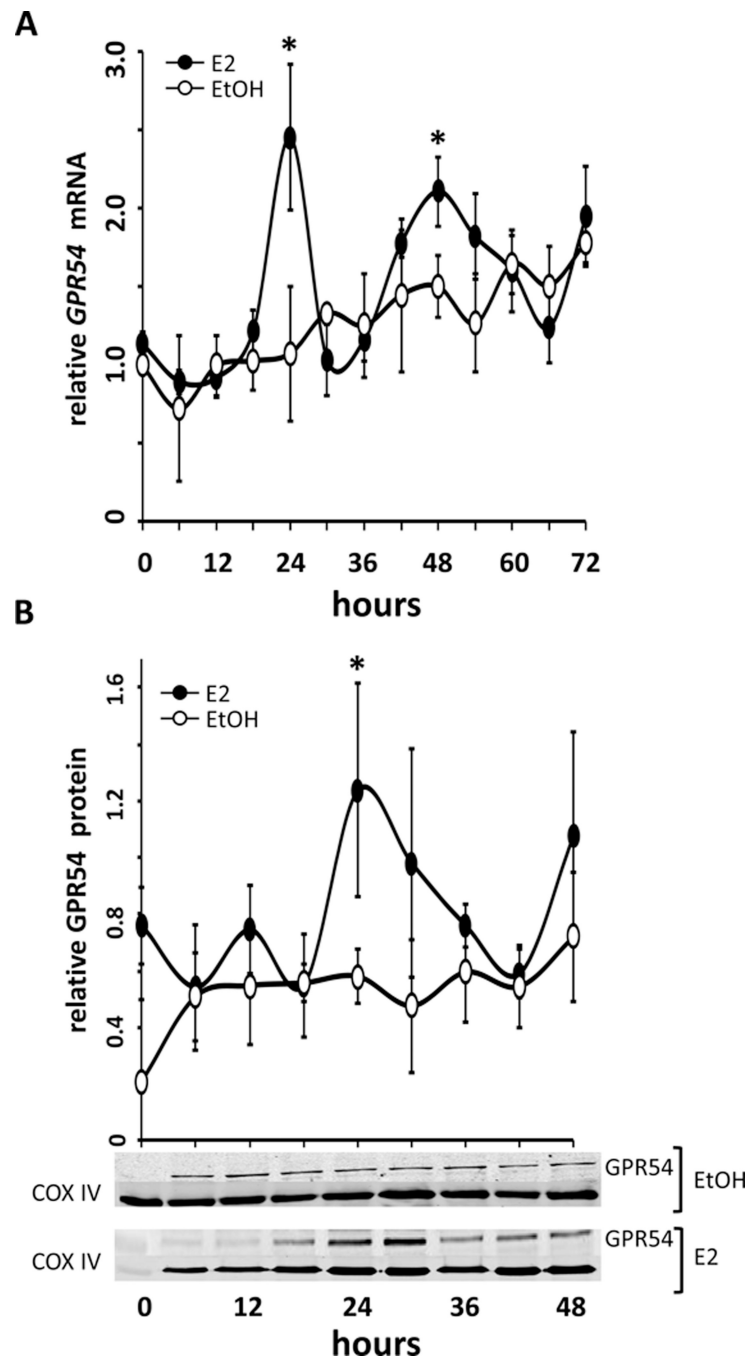
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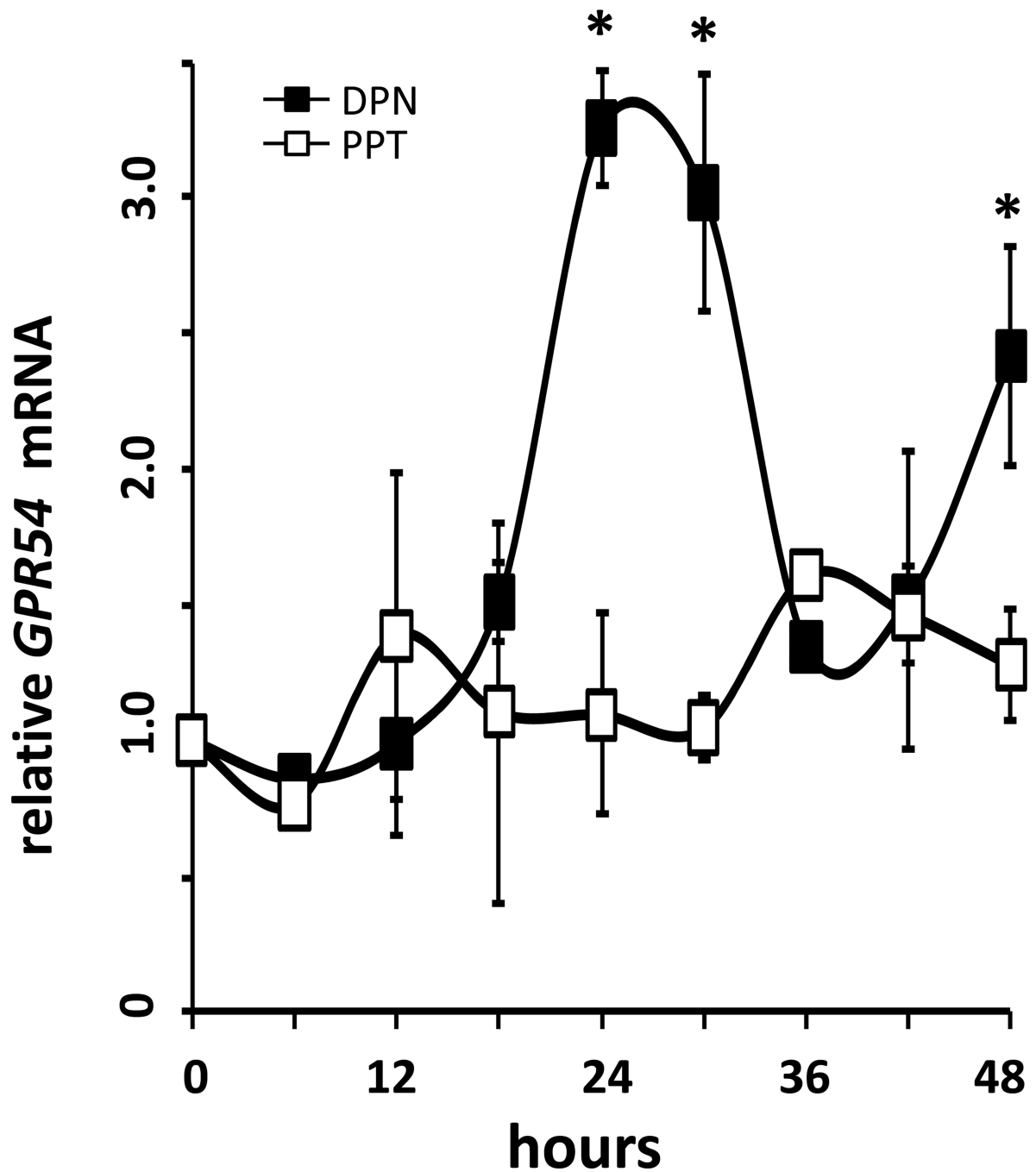
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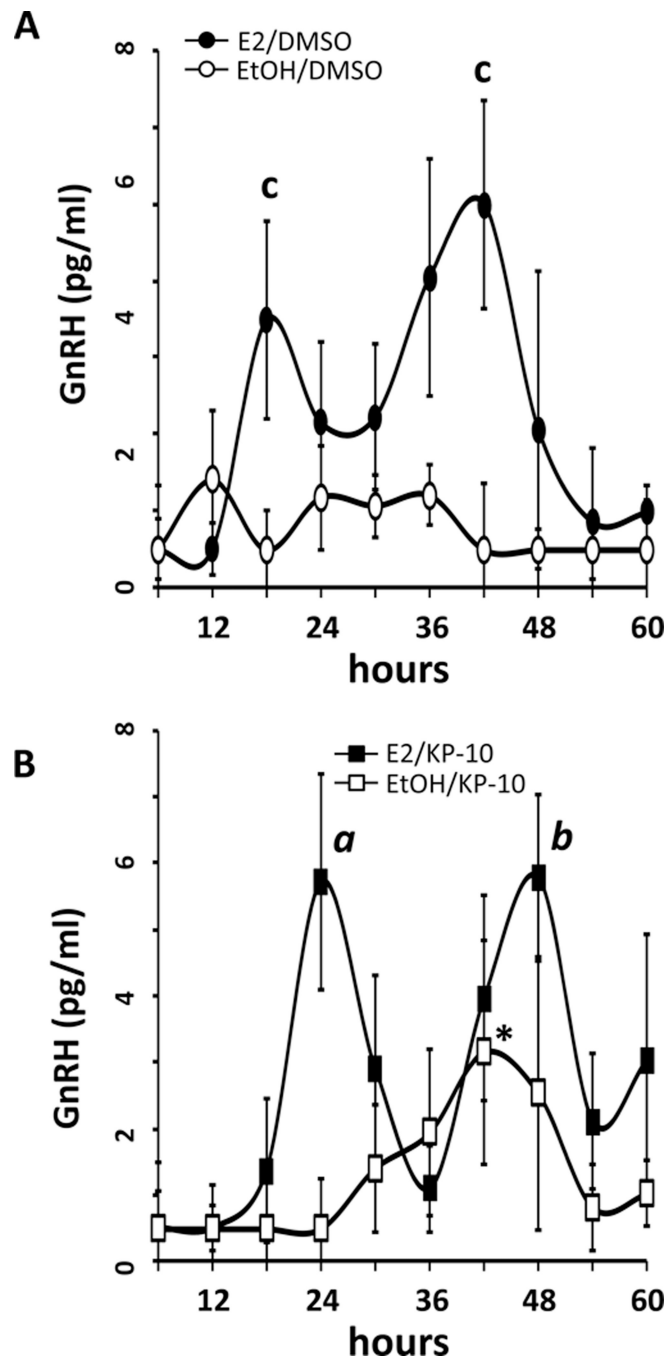


**Figure 1.** *GPR54* mRNA and protein expression in GT1-7 cells exhibits rhythmic expression only in the presence of oestradiol (E2). GT1-7 cells were treated with either 0.05% ethanol vehicle (EtOH, open circles) or 100.0 pM 17 $\beta$ -E2 (E2, filled circles) after serum synchronization. (A) *GPR54* expression levels (mean  $\pm$  SEM) are shown relative to the 0-hour, pre-treatment sample, using real time PCR. The changes in *GPR54* expression occur with an approximate 24h periodicity, with E2-treated GT1-7 *GPR54* abundance highest at 24 hours and rising again at 48 hours (\*,  $P < 0.05$  vs. EtOH,  $n = 4$ ). (B) Protein abundance (mean  $\pm$  SEM) of *GPR54* in GT1-7 cells shows corresponding rhythmic expression patterns. In 17 $\beta$ -E2-treated

cells, (E2, filled circles) GPR54 protein (normalized to COX IV) peaks at 24 hours (\*,  $P < 0.05$  vs. EtOH) and 48 hours, although the latter did not reach statistical significance. Representative western blots show a ~45kDa band of GPR54 with peak levels at 24 and 48 hours, corresponding to average levels in (B), normalized to COXIV (~18kDa).



**Figure 2.** *GPR54* mRNA increases stimulated by  $17\beta$ -E2 is mimicked by the ER $\beta$  agonist DPN, but not the ER $\alpha$  agonist PPT. GT1-7 cells were treated with 200.0pM PPT (PPT, open squares) or 200.0pM DPN (DPN, filled squares) after serum shock. *GPR54* mRNA changes were quantified using real time PCR relative to the 0h time point, prior to treatment. *GPR54* mRNA expression (mean  $\pm$  SEM) showed a rhythmic pattern in DPN-treated cells, with peaks at 24-, 30- and 48 hours ( $P < 0.05$  vs PPT,  $n=4$ ).

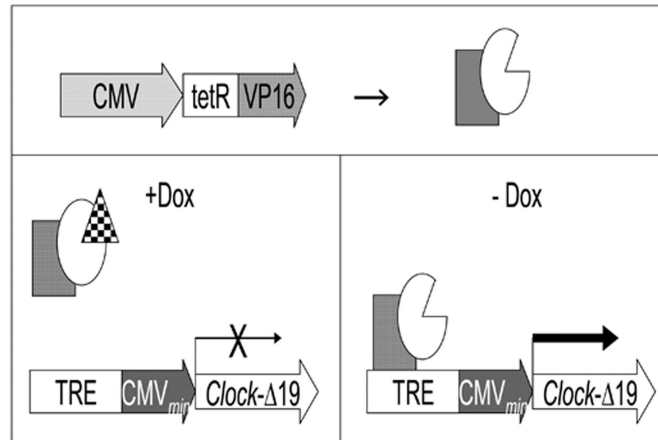
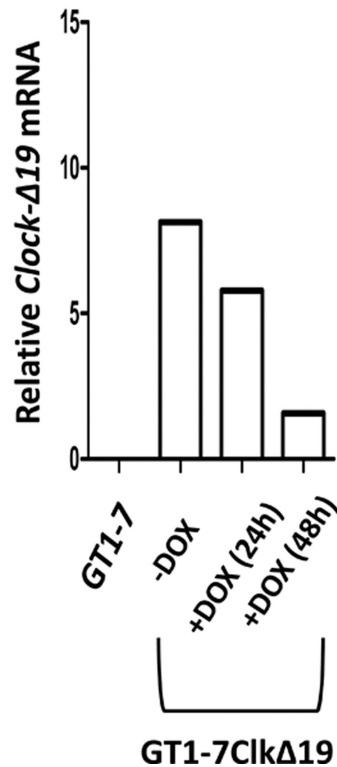
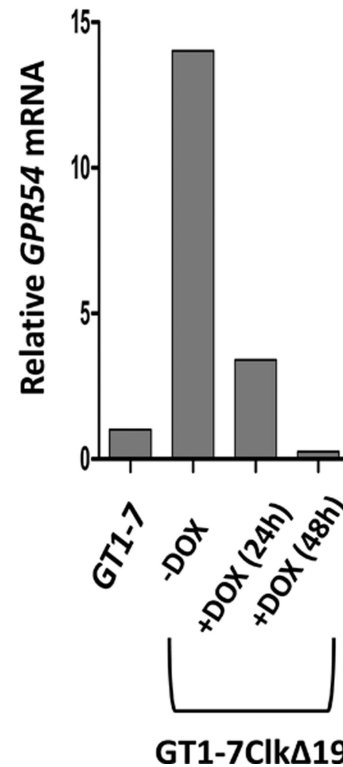


**Figure 3.**

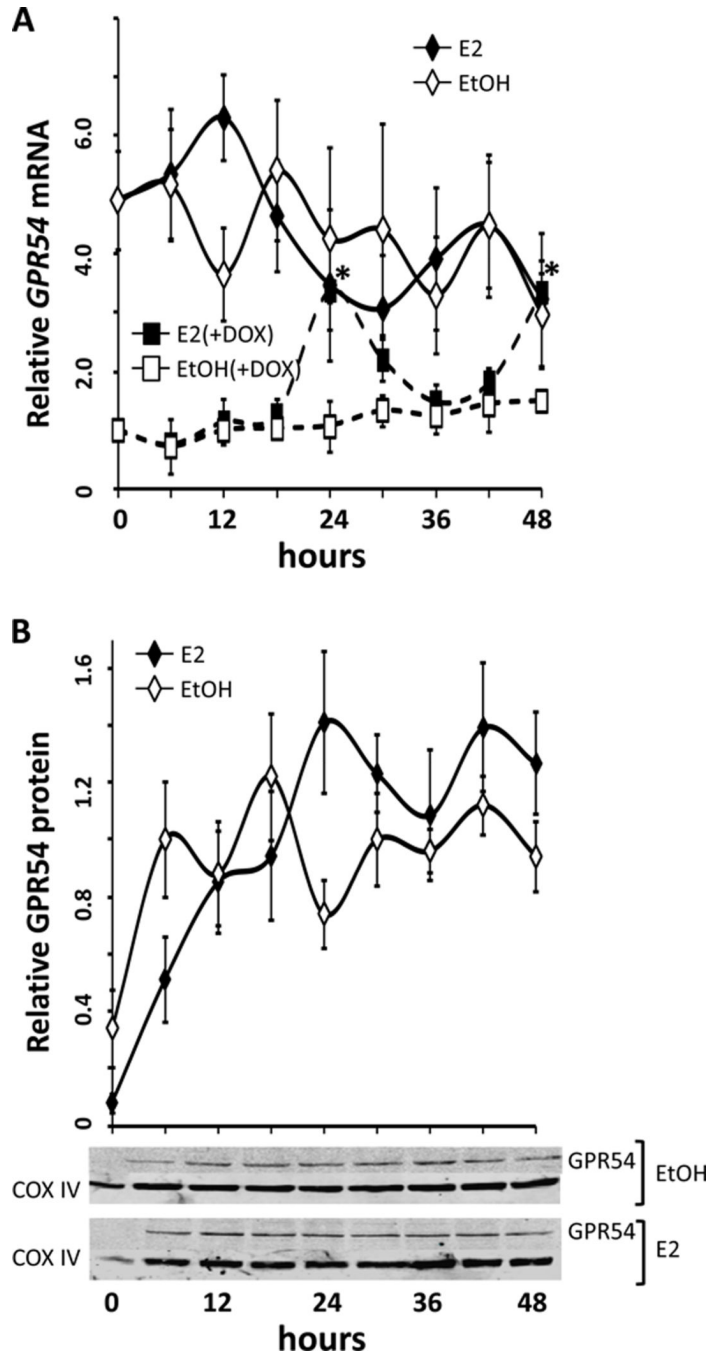
$17\beta$ -E2 treatment potentiates Kisspeptin-induced GnRH secretion from GT1-7 cells. GT1-7 cells treated with 0.05% ethanol vehicle showed constitutively low GnRH release when stimulated with 0.01% DMSO (A; EtOH/DMSO, open circles), whereas GT1-7 cells exposed to 45 min treatments with 10.0 nM Kisspeptin-10 (B; EtOH/KP-10, open squares) produced modest but not significant GnRH release between 30 and 48 hours, except for 42h (\*,  $P < 0.05$  vs. EtOH/DMSO,  $n = 4$ ). In contrast, GnRH release from GT1-7s treated with 100pM E2 and stimulated with DMSO (A; E2/DMSO, filled circles) showed circadian peaks in GnRH release at 18 and 42 hours after serum shock ( $c$ ,  $P < 0.05$  vs EtOH,  $n = 4$ ). GnRH

release from 100pM E2-treated cells (B; E2/KP-10, filled squares, n=4) also exhibited rhythmic release following kisspeptin stimulation, with peaks at 24 and 48 hours (*a*,  $P < 0.05$  vs EtOH/KP-10; *b*,  $P < 0.05$  vs EtOH/DMSO, n=4). Increases in GnRH secretion correspond with increased GPR54 mRNA and protein expression levels (Fig 1.) All values depicted are mean  $\pm$  SEM.



**A****B****C****Figure 4.**

The clock-disrupting *CLOCK-Δ19* was stably transfected into GT1-7 cells using the tet-OFF *in vitro* system. (A) Expression of the dominant negative *CLOCK-Δ19* occurs robustly in the absence of doxycycline (DOX, 10.0  $\mu$ M) treatment. In the presence of DOX, *CLOCK-Δ19* expression is repressed and the endogenous clock functions normally. (B) *Clock-Δ19* mRNA is not present in wild-type GT1-7 cells, but is expressed in this subcloned line and is sensitive to DOX treatment. (C) Additionally, *GPR54* mRNA is elevated in GT1-7 $\Delta$ 19 cells grown in the absence of DOX compared to wild-type GT1-7 cells or DOX-treated GT1-7 $\Delta$ 19 cells.



**Figure 5.**

Induction of the dominant negative CLOCK- $\Delta 19$  in GT1-7 cells abolishes circadian variation in *GPR54* mRNA and protein expression. (A) *GPR54* mRNA expression in E2- (black diamonds,  $n=3$ ) and EtOH- (white diamonds,  $n=3$ ) treated GT1-7Clk $\Delta 19$  cells was elevated, but arrhythmic, in contrast to that observed in “clock-intact” DOX-pre-treated GT1-7 Clk $\Delta 19$  cells following E2 treatment (black squares,  $n=3$ ; \*,  $P<0.05$  vs EtOH, white squares,  $n=3$ ). (B) Average Western blot density ( $n=3$ ) showing *GPR54* protein expression in GT1-7Clk $\Delta 19$  cells treated with E2 or EtOH vehicle. No rhythms in *GPR54* expression were detected in GT1-7Clk $\Delta 19$  cells. A representative western blot shows a ~45kDa band

indicating GPR54, and COXIV loading controls (~18kDa). All values depicted are mean  $\pm$  SEM.