Effects of cycle stage on regionalised galanin, galanin receptors 1–3, GNRH and GNRHR receptor mRNA expression in the ovine hypothalamus

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Abstract

The neurotransmitter galanin has been implicated in the steroidogenic regulation of reproduction based on work mainly conducted in rodents. This study investigated the temporal changes in the expression of galanin and its three receptor isoforms and GNRH and GNRHR mRNA in specific hypothalamic nuclei known to be involved in the regulation of reproductive cyclicity, namely the medial pre-optic area (mPOA), the rostral mPOA/organum vasculosum of the lamina terminalis, the paraventricular nucleus and the arcuate nucleus using an ovine model. Following synchronisation of their oestrous cycles, tissues were collected from ewes at five time points: the early follicular, mid follicular (MF) and late follicular phases and the early luteal and mid luteal phases. The results indicated significant differences in regional expression of most of the genes studied, with galanin mRNA expression being highest during the MF phase at the start of the GNRH/LH surge and the expression of the three galanin receptor (GalR) isoforms and GNRH and its receptor highest during the luteal phase. These findings are consistent with a role for galanin in the positive feedback effects of oestradiol (E₂) on GNRH secretion and a role for progesterone induced changes in the pattern of expression of GalRs in the regulation of the timing of E₂’s positive feedback through increased sensitivity of galanin-sensitive systems to secreted galanin.


Introduction

Substantial evidence shows that the neuropeptide galanin is involved in the regulation of reproductive function in rodents, although its exact role remains to be determined. Galanin signalling is mediated through three membrane-bound receptor isoforms (galanin receptor 1 (GalR1), GalR2 and GalR3) that are immunologically identifiable, functionally distinct and associated with specific mRNA sequences. The receptors are associated with G proteins in the cell membrane, composed of three basic subunits (α, β and γ) and are capable of activation of different intracellular signalling pathways (Brannt et al. 1987, Betty et al. 1998, Straiker et al. 2002). GalR1 and GalR3 are known to activate Gi proteins and are typically considered inhibitory, while GalR2, which is associated with Go/q/11 proteins, is considered stimulatory. Given the reported distribution of galanin (Melander et al. 1986, Skofitsch & Jacobowitz 1986) and its membrane-bound receptors (GalR1, GalR2 and GalR3; Wang et al. 1997, 1998, Smith et al. 1998, Waters & Krause 2000) within the hypothalamus and pituitary gland, roles both as a neurotransmitter and as a neuromodulator have been proposed (Lopez & Negrovilar 1990, Merchenthaler et al. 1990, Cheung et al. 1996, Gajewska et al. 2004, Merchenthaler 2005). Specifically, it has been hypothesised that galanin could play a role in oestrogen’s positive feedback effects on gonadotropin-releasing hormone/luteinising hormone secretion, as its expression is positively regulated by oestrogen (Shen et al. 1998). Studies in sheep have reported that galanin is expressed in similar regions within the ovine hypothalamic–pituitary axis as laboratory rodents (Chaillou et al. 1999). GalR1 and GalR2 have also been shown to be expressed in the ovine hypothalamus, although the patterns and levels of expression differ from those reported in rodents (Dufourny & Skinner 2004, Chambers et al. 2007). In this regard, it should be noted that while there are aspects of reproductive physiology that are common between rodents and sheep, there are also many significant differences, e.g. sheep are seasonally reproductively active, ovulation is independent of circadian effects and rodents show a much abbreviated luteal phase. The aim of this study was to determine the effects of exposure to different physiologic (steroidogenic) states on the expression...
of the mRNAs for galanin and its three receptor isoforms within the hypothalamus of the sheep and more specifically to characterise changes in expression of these genes relative to the changes in GNRH and GNRH receptor mRNA expression during different stages of the ovine oestrous cycle within specific hypothalamic nuclei.

Materials and Methods

Animals and tissue collection

The study was conducted using 30 Scottish mule ewes maintained under normal husbandry conditions at the Cochno Research Facility, University of Glasgow, UK. Briefly, to allow characterisation of mRNA expression at defined time points, the oestrous cycles of the study groups were synchronised by treatment with exogenous progesterone (2X controlled intravaginal drug release (goat) devices (CIDR) InterAg, Hamilton, New Zealand) for 14 days (cycle 1 in Fig. 1). Twelve ewes were killed relative to the time of CIDR removal such that tissue was collected from six ewes during the early luteal (EL; 6 days after CIDR removal, progesterone rising, GNRH/LH decreasing) and mid luteal (ML; 10 days after CIDR removal, high progesterone, low GNRH/LH) phases (cycle 2 in Fig. 1).

To ascertain the timing of the endogenous pre-ovulatory LH surge that occurred following CIDR removal (cycle 1 in Fig. 1), the remaining 18 ewes were blood sampled at 4 h intervals between 24 and 36 h, 2 h intervals from 38 to 56 h, 4 h intervals from 56 to 64 h after CIDR withdrawal, with a final sample collected 8 h later. Animals were then treated with further two CIDRs for 14 days to allow synchronisation of the subsequent follicular phase (cycle 2 in Fig. 1). Animals were killed and tissues were collected 12 (n=6), 40 (n=6) and 60 h (n=6) after removal of the second CIDRs. The allocation of animals and timing of tissue collection were based on LH data obtained in cycle 1 and were targeted to procure tissues during the early follicular (EF; 12 h after CIDR removal, no progesterone, low E2, low GNRH), mid follicular (MF; pre-early LH surge; no progesterone, high E2, GNRH rising) and late follicular (LF; after the LH surge; E2 and low progesterone, low LH, high GNRH) phases. Blood samples were obtained from these 18 ewes at appropriate intervals (combination of 2 and 4 h samples) following CIDR removal until killed to characterise changes in LH and progesterone secretion as a monitor for cycle stage. All animals were killed with a lethal dose of barbiturates (20 mg/kg BW i.v.; Lethobarb, Duphar Vet, Southampton, Hants, UK). Immediately following death, the brains were removed and a tissue block containing the pre-optic area (POA)/hypothalamus was dissected out, bisected along the midline and frozen at −70°C until processed.

All procedures were approved by the Faculty of Veterinary Medicine’s Ethics and Welfare Committee, University of Glasgow and were carried out in accordance with the UK Animal Scientific Procedures Act.

Tissue processing

Hemi-hypothalami were mounted in a dissecting matrix and 2 mm slices were harvested. To achieve consistency in tissue analysis between animals, tissue was harvested relative to

![Figure 1](https://example.com/figure1.png)  
**Figure 1** Experimental design utilised for the main study. Five groups of ewes were run through one or two artificial luteal phases by treatment with exogenous progesterone (presence of controlled intravaginal drug release devices (CIDR), indicated by the grey bars). Blood samples were collected during the follicular phase that followed CIDR removal in cycle 1, in all animals, to allow determination of the timing of the leutinising hormone (LH) surge (white box). Ewes were killed (X and vertical dashed line) at times corresponding to the early (EL) or mid (ML) luteal phase (cycle 2, no exogenous progesterone treatment) or during the early (EF, mid (MF) or late (LF) follicular phase (cycle 2). Additional blood samples were collected from ewes during cycle 2 to characterise progesterone secretion (EL and LL) and progesterone and LH secretion (EF, MF and LF) groups.
clearly observable anatomical landmarks. Coronal slices were cut relative to the anterior commissure (visible in all tissue blocks), such that one slice encompassed the anterior commissure (which in cross section is ~2 mm wide in the ewe), additional 2 mm slices were taken both forwards and backwards from the slice containing the anterior commissure. Two millimetre diameter punches were then recovered from slices using neuroanatomical landmarks and brain topography as described in other species (Palkovits 1973, Briski et al. 2009, Ely et al. 2011), such that tissue collection was standardised across animals and targeted specific hypothalamic nuclei, namely the medial POA (mPOA), rostral mPOA/or ganum vasculum of the lamina terminalis (OVLT), superior aspect of the caudal OVLT, bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), anterior hypotalamic area (AHA), supraoptic nucleus (SON), entromedial nucleus (VMN), arcuate nucleus (ARC) and the dorsomedial nucleus.

**mRNA quantification**

Total RNA was extracted from the tissue using Trizol (Invitrogen) according to the manufacturer’s instructions. cDNA was obtained by RT of the resultant mRNA using random hexamers (Promega), Moloney murine leukaemia virus reverse transcriptase (Invitrogen) and Rnasin (Promega) as described previously (Oshaughnessy & Murphy 1993). mRNA and cDNA purity and quantity were assessed by spectroscopy. An approximate yield of 1000 ng/μl was achieved for most samples. Quantitative PCR (qPCR, Stratagene Mp3000; Amplitaq Gold, Applied Biosciences) for galanin and the three receptor isoforms GalR1, GalR2 and GalR3 and for GNRH and GNRHR was performed using the primers, probes and procedures described previously (Chambers et al. 2007, Whitelaw et al. 2009). qPCR was completed on all punches from ewes in the ML and LF phase groups (n = 12). Each of the measured mRNAs was expressed in all areas of hypothalamus studied (data not shown). The areas of the hypothalamus that showed the highest overall levels of gene expression were the mPOA (GNRH), mPOA/OVLT (galanin, GalR1, GalR3, GNRH and GNRHR), PVN (GalR2) and ARC (GalR1 GalR2, GalR3 and GNRHR); low undetectable gene expression was found in some of the other areas. Based on the results obtained from these two groups, qPCR analysis was rationalised in the remaining groups to focus on these four selected areas.

**Hormone concentrations**

LH concentrations were measured in duplicate using 100 μl aliquots of plasma using a previously described, double-antibody RIA (Niswender et al. 1969) with LH standard NIDDK-oLH-I-3 and antiserum NIDDK-anti-oLH-1 (NIDDK, Torrance, CA, USA). Intra- and inter-assay coefficients of variation averaged were 7.98 and 8.22% respectively. Sensitivity averaged 0.29 ng/ml. Concentrations of progesterone were determined in a single assay using a commercially available kit (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA, USA) with a limit of detection of 0.10 ng/ml.

For relative quantification of mRNA concentrations, the comparative Ct method was used, wherein the expression of each gene of interest was quantified relative to the expression of the housekeeping gene (β-actin; user bulletin no. 2, PE Biosystems, UK). Results were natural log (Ln) transformed to equalise variance and were multiplied by 10 000 for ease of data handling. All results are expressed as mean ± S.E.M.

The effects of hypothalamic area and cycle stage (EL, ML, EF, pre- and post-LH surge) on gene expression were compared using ANOVA (Genstat, release 10; VSN International Ltd, Hemel Hempstead, UK). To equalise variance, the data were Ln transformed before analysis and differences between treatment groups were calculated using least significant difference analysis. Significance was set at P < 0.05.

**Results**

**Hormone concentrations**

Mean progesterone concentrations in the two groups of animals in which tissues were collected in the luteal phase (EL and ML) are presented in Fig. 2a. Progesterone concentrations in the two luteal phase groups were low 2 days after CIDR withdrawal but increased over the course of the following 8 days, reaching maxima of 2.6 and 4.8 ng/ml before killing of the EL and ML phase animals respectively.

Progesterone and LH concentrations observed during the EF phase are presented in Fig. 2b. Progesterone concentrations on the day before CIDR removal (13 days after CIDR insertion) averaged 8.2±1.3 ng/ml and decreased precipitously following CIDR removal. Mean LH concentrations were low before CIDR removal. A transient increase in LH was evident immediately after CIDR withdrawal, coincident with the fall in circulating progesterone concentrations.

Mean LH concentrations in the MF and LF phase groups are shown in Fig. 2c. In the MF phase group, LH concentrations tended to increase in the first 20 h after CIDR removal but remained relatively stable thereafter until the last sample where an increase was evident. This increase in the mean LH concentration in the MF phase group was principally due to one animal in which an elevated LH concentration was observed, suggesting the animal was beginning an LH surge. A sustained increase in LH, indicative of an LH surge, as defined previously (Caraty et al. 1995), was seen in all of the animals in the LF phase group. The average time of LH surge onset in these ewes was 43-3-1.0 h after CIDR removal. The peak of the LH surge (mean 25-21 ± 4.46 ng/ml) was seen in all but one of the ewes (on average 47-6 ± 2.2 h) after CIDR removal. In the remaining ewe, the highest LH concentration was observed in the last sample collected (58 h after CIDR removal).
Gene expression

Effect of hypothalamic area Mean levels of expression of galanin and its three receptor isoforms, across the five stages of the cycle, in the four areas studied are presented in Fig. 3. ANOVA revealed a significant effect of hypothalamic area on galanin mRNA expression ($P<0.01$), with expression being significantly higher in the mPOA/OVLT, relative to both the mPOA and the ARC.

Considerable inter-animal variation was observed in GalR isoform mRNA expression across both hypothalamic area and cycle stage. ANOVA did not identify significant effects of hypothalamic area on mRNA expression for any of the three receptor isoforms; however, a trend was noted for variation in GalR1 ($P=0.089$) and GalR3 ($P=0.094$) mRNA expression with hypothalamic areas. These trends were associated with lower overall expression in the mPOA for all three receptor isoforms (GalR1, 1.8-fold; GalR2, 2.7-fold; GalR3, 2.6-fold).

Mean GNRH and GNRHR mRNA expression levels across hypothalamic areas studied and cycle stage are shown in Fig. 4. GNRH mRNA expression was significantly affected by hypothalamic area ($P<0.05$), with the highest expression occurring in the mPOA and the mPOA/OVLT. In contrast, while GNRHR mRNA expression also differed significantly ($P<0.005$) with hypothalamic areas, the main effect noted was lower expression within the mPOA relative to the other areas studied.

Effect of cycle stage Galanin mRNA expression was not significantly affected by cycle stage; however, there was a trend ($P=0.10$) for a cycle stage by area interaction, due to low galanin mRNA expression in the mPOA throughout the cycle and increased expression in the mPOA/OVLT, PVN and ARC, particularly during the MF phase.

GalR1 mRNA expression was significantly ($P<0.05$) influenced by cycle stage, with expression being significantly higher in the EL phase when compared with the LF phase (all areas studied). Additional statistically significant differences were present between the EL and the EF phases of GalR1 mRNA expression in the mPOA and mPOA/OVLT. These effects of cycle stage were accompanied by a trend for variation in GalR1 expression between hypothalamic areas (see above), but there was no cycle stage by area interaction.

GalR2 mRNA was expressed at significantly ($P<0.05$) higher levels throughout the luteal phase compared with both the EF and LF phases. Luteal GalR2 mRNA expression was not different compared with that seen in the MF phase as mRNA expression was consistently, although not significantly, higher across the four hypothalamic areas studied in the MF phase compared with both the EF and the LF phases.

In general, patterns of GalR3 mRNA expression across the cycle were similar to GalR2, although the absolute levels of expression were greater for GalR3. GalR3 mRNA expression was significantly ($P<0.05$) influenced by cycle stage, expression again being higher in the luteal phase compared with EF and LF phases.
GNRH mRNA expression was significantly affected by cycle stage \((P<0.05)\) wherein expression levels were significantly higher during the EL phase than both the EF and the LF phases and were higher in the ML phase than the LF phase.

GNRHR mRNA expression was not significantly affected by cycle stage, but a strong trend \((P<0.06)\) was noted for higher levels of expression during the luteal phase compared with the follicular phase, in particular within the mPOA and mPOA/OVLT.

**Discussion**

Findings from this study indicate that the hypothalamic expression of mRNA for galanin and its three receptors (GalR1, GalR2 and GalR3) changes in a region and/or cycle-dependent manner in the ewe. Interestingly, levels of mRNA expression for galanin and its receptors changed in opposite directions. Furthermore, regulation of mRNA expression for the three GalR isoforms appears coordinated despite their possible alternate cellular actions. Given the patterns of expression of GNRH, galanin and their receptors within the hypothalamus, it has been proposed that galanin could transduce oestrogenic signals to GNRH neurons, which in themselves do not express oestradiol (E2) receptor \(\alpha\) (Herbison et al. 1993, Lehman et al. 1993). The results of this study indicate that responsiveness of neural systems to galanin (mRNA expression for the GalR isoforms) in areas of the hypothalamus known to be important for either the direct or the indirect stimulation of GNRH secretion increases over the luteal phase, when galanin mRNA expression remains low. These changes in GalR mRNA expression paralleled changes in mRNA for both GNRH and its receptor. These changes are of interest if galanin is indeed important for the transduction of oestrogen–positive feedback to GNRH neurons, as previous work in sheep has shown that sensitivity to E2 is influenced by progesterone pre-exposure (Skinner et al. 2000). The luteal phase increase in galanin responsivity was followed by a rise in galanin mRNA expression that, in this study, peaked at the time of the LH surge. The data, therefore, support the proposal that galanin could affect the actions of E2 to stimulate GNRH secretion during the follicular phase in the sheep, as has been proposed in the rat (Finn et al. 1998).
The results of the current study demonstrate that galanin mRNA is expressed in a variety of hypothalamic nuclei (including the caudal mPOA and ARC) confirming immunocytochemical (Chaillou et al. 1999, Dufourny et al. 2003, Tourlet et al. 2005, Qi et al. 2008) and molecular studies (Henry et al. 2001, Bellingham et al. 2010). Similarly, GNRH mRNA was shown to be highly expressed in the mPOA and mPOA/OVLT and be detectable in the ARC and PVN, as reported using both immunocytochemical (Lehman et al. 1986, Caldani et al. 1988, Dudas & Merchenthaler 2006) and molecular approaches (Harris et al. 1998, Robinson et al. 2000). This study extends previous work by showing that the hypothalamic areas that contain galanin and GNRH mRNA also express mRNA for the three GalRs and, thus, provide the mechanistic links required for galanin to be able to regulate GNRH secretion. Such a role for galanin had previously been proposed given the presence of GalR1 mRNA containing cells in regions of the hypothalamus that contain GNRH (Faure-Virelizier et al. 1998, Landry et al. 1998, Dufourny & Skinner 2004, Chambers et al. 2007) and the observation that galanin is co-expressed within GNRH neurons in rodents (Coen et al. 1990, Merchenthaler et al. 1990) and sheep (Dufourny et al. 2003). While the results of this study indicate GalR2 mRNA expression in these same hypothalamic nuclei, as we have previously shown that E2-responsive hypothalamic cells that are immunocytochemically positive for GalR2, do not co-localise with GNRH in the sheep (Chambers et al. 2007), direct regulatory effects of galanin on GNRH secretion are unlikely to be mediated by GalR2. Given its distribution within the hypothalumus and pituitary, GalR3 has been proposed to be involved in the regulation of a variety of physiological functions including food intake, fluid homeostasis, cardiovascular function and nociception (Mennicken et al. 2002). In this study, GalR3 mRNA was expressed in the four hypothalamic nuclei studied (Smith et al. 1998, Waters & Krause 2000, Mennicken et al. 2002), but, unlike previous reports, GalR3 mRNA was expressed at a similar or at higher level than GalR1 and GalR2 in some nuclei (mPOA, PVN and ARC), suggesting a link to reproductive function.

Previous work has reported that hypothalamic galaninergic gene expression varies with oestrous cycle stage (Brann et al. 1993, Rossmannith et al. 1996, Finn et al. 1998, Tourlet et al. 2005) and/or is affected by steroids (Tourlet et al. 2005). Using a targeted approach, the results of this study suggest that galanin mRNA expression changes over the oestrous cycle, in a region-specific manner (mPOA/OVLT, PVN, ARC but not mPOA), conducive with localised actions. These results contrast with a previous study in which hypothalamic galanin expression did not change with either reproductive state or oestrous cycle stage (Dufourny et al. 2003). That study, however, used immunocytochemistry and it is possible that more localised transitory changes in expression could have been missed. Elevated galanin expression during the follicular phase has previously been reported in rodents (Brann et al. 1993, Rossmannith et al. 1996, Finn et al. 1998) and in one study on sheep in which galanin expression was assessed as the number of detectable immunoreactive cells (Tourlet et al. 2005). While Tourlet et al. (2005) reported changes in ovine galanin expression within the POA but not in the BNST or infundibular nucleus in response to an E2 signal, comparison of the anatomical data indicates that the stimulatory effects on galanin immunoreactive cells corresponds with the increase in mRNA expression seen in the mPOA/OVLT in the current study, with little effect in the more rostral regions of the POA.

Expression of mRNA for the three GalR isoforms was found to be significantly affected by oestrous cycle stage, being highest during the luteal phase. This suggests that GalR mRNA expression could be positively regulated by progesterone, a result that is supported by the high levels of hypothalamic GalR mRNA expression seen in pregnant compared with luteal and follicular phase ewes (C M Whitelaw, J E Robinson, P M Hastie, V Padmanabhan and N P Evans, unpublished observation) and the dynamic changes in mRNA expression seen across the luteal phase in this study. Previous reports, principally in rodents, have indicated that GalR1 mRNA expression varies with cycle stage and in response to E2 or E2 and progesterone (Faure-Virelizier et al. 1998, Mitchell et al. 2004). While the results of such reports are at times contradictory, they...
suggest that hypothalamic GalR1 mRNA expression is regulated in a region and cell population-specific manner (Faure-Virelizier et al. 1998, Mitchell et al. 2004). The apparent difference relative to the current results could be due to the fact that the rodent studies concentrated on the follicular phase, where progesterone has a facilitatory effect on E2-induced LH secretion, whereas the changes observed in the current study relate to the luteal phase, during which the effects of progesterone on the reproductive neuroendocrine axis are entirely inhibitory. This study provides novel data relative to the changes in hypothalamic GalR2 and GalR3 mRNA expression, as little has been published in this regard either across the oestrus cycle or in response to gonadal steroids in the female. While a study in male rats reported that GalR2 mRNA expression in the ARC is inhibited by testosterone (Bouret et al. 2000), the exact role played by the different GalR isoforms in the regulation of neural function and specifically GNRH secretion, either directly or indirectly, in either sex, remains to be determined. While GalR1 and GalR3 are typically considered stimulatory and GalR2 inhibitory, it has recently been shown that the β/γ protein subunits of the receptors, which dissociate from the α-subunit upon ligand binding, can activate and/or inhibit additional patterns of intracellular signalling (Wang et al. 1998). The actions of the three different GalRs may therefore be highly complex and could indeed be specific to certain cell populations.

The results of the current study indicated relatively constant GNRH mRNA expression across the oestrus cycle in contrast to those reported previously in sheep, where a decrease was observed over the follicular phase (Harris et al. 1998, Robinson et al. 2000). It should be noted, however, that the timing of tissue collection differed between these studies. Relative to the current results, it is interesting to note that while an earlier ovine study reported no significant relationship between progesterone and hypothalamic GNRH mRNA expression (assessed as mean silver grain density per cell; Robinson et al. 2000), they did report a non-significant increase in the number of cells that expressed GNRH mRNA between ovariectomised animals and those treated with EL or ML phase concentrations of exogenous progesterone. Thus, despite the fact that the study by Robinson et al. used ovariectomised ewes treated with exogenous progesterone and the current study was conducted using ovary-intact animals during the natural cycle, the results from both studies suggest increasing expression of GNRH mRNA under conditions of rising or elevated progesterone. Comparison of the changes in GNRH mRNA expression over the course of the oestrous cycle in the ewe with those reported in laboratory rodents is inherently difficult, due to both species differences in progesterone action indicated above and circadian influences as outlined by Robinson et al. (2000).

Changes in GNRHR expression with cycle stage have been well characterised in the pituitary gland, where in general it is thought to be positively regulated by E2 (Rispoli & Nett 2005). Data on other tissues, however, would suggest that GNRHR may be differentially affected by steroids and tissue location (Ban et al. 1990). It has been reported that GNRHR is expressed within the mammalian hypothalamus and that, in the rat, hypothalamic GNRHR mRNA increases before the LH surge under the influence of E2 (Jennes et al. 1997). The results of the current study indicated that, in sheep, GNRHR mRNA was highest during the luteal phase, when progesterone was elevated, and lowest during the LF phase. As with the ligand, this result could suggest species differences in the steroidogenic regulation of hypothalamic GNRH mRNA expression that could again reflect the different actions of progesterone in rodents and sheep.

In conclusion, the results of this study support the steroidogenic regulation of GNRH and GNRHR mRNA in the hypothalamus of the ewe over the course of the oestrous cycle. In addition, it would appear that the expression of both galanin and its three reported receptor isoforms are also steroid sensitive. As galanin mRNA was seen to change in the MF phase, and the mRNA for its receptors was elevated in the luteal phase, it would appear that expression of the ligand and receptor mRNA may be regulated by E2 and progesterone respectively. In addition, the time course of the observed changes in mRNA expression agrees with a progesterone-driven increase in sensitivity of galanin-responsive neural systems during the luteal phase in readiness for an E2-driven increase in galanin signalling at the time of the LH surge.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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