

Co-expression of c-Fos with oestradiol receptor α or somatostatin in the arcuate nucleus, ventromedial nucleus and medial preoptic area in the follicular phase of intact ewes: alteration after insulin-induced hypoglycaemia.

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Abstract

The aim of this study was to investigate how acute insulin-induced hypoglycaemia (IIH) alters the activity of cells containing oestradiol receptor α (ER α) or somatostatin (SST) in the arcuate nucleus (ARC) and ventromedial nucleus (VMN), and ER α cells in the medial preoptic area (mPOA) of intact ewes. Follicular phases were synchronised with progesterone vaginal pessaries. Control animals were killed at 0h or 31h (n=5 and 6, respectively) after progesterone withdrawal (PW; time zero). At 28h, 5 other animals received insulin (INS; 4 iu/kg) and were subsequently killed at 31h. Hypothalamic sections were immuno-stained for ER α or SST each with c-Fos, a marker of neuronal transcriptional activation. Insulin did not alter the percentage of activated ER α cells in the ARC, however, there was circumstantial evidence to indicate that two insulin-treated animals (INS responders, usually with suppressed LH surge) had an increase in the VMN (from 32 to 78%) and a decrease in the mPOA (from 40 to 12%) compared to no increase in the two INS non-responders (usually with LH surge). The percentage of activated SST cells in the ARC was greater in all four insulin-treated animals (from 10 to 60%), whereas there was circumstantial evidence to indicate that activated SST cells in the VMN increased only in the two insulin-responders (from 10 to 70%). From these results, we suggest that IIH stimulates SST activation in the ARC as part of the glucose-sensing mechanism but ER α activation is unaffected in this region. We present circumstantial evidence to support a hypothesis that disruption of the GnRH/LH surge may occur in insulin responders via a mechanism that involves, at least in part, SST cell activation in the VMN along with decreased ER α cell activation in the mPOA.

Introduction

The ovarian steroid hormone oestradiol is of central importance in the control of reproductive neuroendocrine function in female mammals. For the greater part of the ovarian cycle in ewes, oestradiol and progesterone act synergistically to restrain gonadotrophin releasing hormone/luteinising hormone (GnRH/LH) secretion through negative feedback action. However, during the late follicular phase, there is a 'switch' from inhibition to enhancement of GnRH secretion (Evans et al. 1995; Karsch et al. 1997). This constitutes oestradiol positive feedback and triggers the onsets of GnRH/LH surge secretion.

The action of oestradiol upon the mammalian brain occurs mainly through classical transcriptional action, namely oestrogen receptor alpha (ER α) signalling (McEwen et al. 2012, Cheong et al. 2014). However, steroid hormone signals do not impinge directly on GnRH cells as these cells do not possess progesterone receptors (PR) or ER α (Shivers et al. 1983; Skinner et al. 2001). Some GnRH neurones express, ER β (Hrabovszky et al. 2001) although it is unlikely that ER β plays a major role in the feedback regulation of GnRH/LH secretion, because ER β knock-out mice have normal fertility (Lubahn et al. 1993; Cheong et al. 2014).

Acute activation of the hypothalamus-pituitary-adrenal axis in the late follicular phase by insulin-induced hypoglycaemia (IIH) lowers plasma oestradiol concentrations and delays the onset of the LH surge in intact ewes (Fergani et al. 2012). Immunohistochemical analysis of c-Fos protein expression (a marker of neuronal transcription activation; Hoffman et al. 1993) revealed that this disruption involved the activation of unknown cell types located in the VMN, ARC and mPOA (Fergani et al. 2014) possibly involving inhibition of ER α -cell activation.

Contrary to our original hypothesis, we have recently shown that there is no inhibition of kisspeptin cell activity in the ARC after a bolus injection of insulin during the late follicular phase (Fergani et al. 2014). Therefore, it seems unlikely that the mechanism for IIH suppression of the LH surge involves kisspeptin cells and alternative pathways merit investigation. In this regard, somatostatin (SST) immunopositive cell bodies are abundant in the VMN and ARC along with SST fibres (but no cell bodies) in both these areas as well as in the median eminence and mPOA (Willoughby et al. 1995; Robinson et al. 2010). Short-

term oestradiol treatment in progesterone-primed ovariectomised ewes increases SST activation in the VMN approximately 10 h before the anticipated onset of an LH surge (Pillon et al. 2004; Robinson et al. 2010). Conversely, in rats, SST is one of the most potent inhibitors of electrical excitability of GnRH neurones identified thus far (Bhattarai et al. 2010) and SST inhibits the LH surge when administered centrally (Van Vugt et al. 2004). Interestingly, recent evidence suggests that hypothalamic SST is also implicated in glucose metabolism by initiating a cascade of events that lead to a peripheral increase in glucose and decrease in insulin (Yavropoulou et al. 2014). It is, therefore, possible that SST cells are activated during insulin-induced disruption of the LH surge and provide an important link between metabolism and reproduction.

In the present study, we examined brain tissue of intact ewes sacrificed in the follicular phase with or without the administration of insulin. Our aim was to determine the effect of IIH on the patterns of ER α and SST transcriptional activation (by measuring co-localisation with c-Fos) in the VMN and ARC, and ER α transcriptional activation in the mPOA, and compare these with peripheral plasma LH, cortisol, progesterone and oestradiol concentrations.

Materials and Methods

Animals, study design, tissue collection, blood collection and hormone assays.

Fifteen adult, ovary-intact Lleyn crossbred ewes were used in the mid-breeding season (3 groups of 5-6 ewes per group). All procedures were conducted within requirements of the UK Animal (Scientific Procedures) Act 1986, and approved by the University of Liverpool Animal Welfare Committee. Frozen coronal sections (40 μ m) used in this study were obtained from the same tissue blocks as described in a previous study on kisspeptin and corticotrophin releasing factor receptor; full details are given in Fergani et al. (2014). Briefly, after follicular phase synchronisation, 5 ml blood was collected via indwelling jugular catheters at 0 h (progesterone intravaginal device withdrawal; PW), 16 h, 24 h and subsequently at 2 h intervals. At 28 h, ewes received 2 ml saline vehicle, or insulin (neutral zinc bovine insulin, Hypurin Neutral, CP Pharmaceuticals, Wrexham UK; i.v. dose of 4 iu/kg body weight). Control animals were killed at 0 h (n=5) and others at 31h after PW (i.e., 3 h after vehicle or insulin administration; control, n=6; insulin, n=5). The insulin dose chosen is routinely used in our studies and evokes a robust cortisol increase and attenuation of the LH

surge (Saifullizam et al. 2010; Fergani et al. 2012). Plasma hormone changes for these ewes are presented in the current study for completeness; full method details appear in Fergani et al. (2014).

Tissue collection

Euthanasia was carried out with sodium pentobarbitone containing 25,000 IU heparin; full details of fixation (Zamboni; picric acid, paraformaldehyde and sucrose) and preservation (at -80 °C) of tissues are given in (Fergani et al. 2014). Free-floating (40 µm) coronal sections were stored in cryoprotectant solution and stored at -20 °C until processed for immunohistochemistry.

c-Fos and ERα or SST dual-label immunofluorescence

All tissue preparation, staining procedures, photography and counting of cells were carried out at the same time as ewes treated with endotoxin (lipopolysaccharide from *E coli*; Fergani et al. 2015) to enable direct comparisons in the Discussion. The observer was unaware of animal identity or group.

Details of the c-Fos methodology (antibody AB-5, PC38, Calbiochem, Cambridge, MA, USA; at a dilution of 1:5000) have already been described (Fergani et al. 2013). This was modified in the present study by co-incubating the polyclonal rabbit anti c-Fos antibody for 72 h with a monoclonal mouse anti-ERα antibody (ID5, M7047, Dako, Carpinteria, CA, USA) at a dilution of 1:50. After incubation, sections were washed thoroughly and incubated with a mixture of donkey anti-rabbit Cy3 (711-165-152, Jackson ImmunoResearch, West Grove, PA) and donkey anti-mouse DyLight 488 (715-485-151, Jackson ImmunoResearch, West Grove, PA) both diluted at 1:500 for 2 h. Thereafter, sections were washed with PBS followed by a final wash with double-distilled water, mounted on chrome alum gelatine coated slides and cover-slipped with Vectashield anti-fading mounting medium (Vector Laboratories Ltd, UK, H-1000).

For c-Fos/SST, a two-step procedure was used. After 72 h incubation with anti-rabbit AB-5 followed by 2 h with anti-rabbit Cy3 to locate c-Fos, a second immunofluorescence procedure was performed: anti-rabbit somatostatin-14 serum (T-4103, Peninsula

Laboratories, San Carlos, CA, at a dilution of 1:500) was incubated for 72 h at 4 °C and then visualised using donkey-anti-rabbit Dylight 488 (715-485-152, Jackson Immunoresearch West Grove, PA), at a dilution of 1:500.

The c-Fos (Ghuman et al. 2011), ER α (Dufourny and Skinner 2002) and SST (Robinson et al. 2010) antibodies have been validated for the use in ovine neural tissue. In addition, negative controls that omitted one of the primary antibodies completely eliminated the appropriate fluorescence without noticeably affecting the intensity of the other fluorescent probe.

Data analysis

Hormone and immunohistochemistry data were analysed with Minitab® 15 statistical package (MINITAB Inc, Pennsylvania, USA). Statistical significance was accepted when $p < 0.05$.

Histological sections were examined under an epi-fluorescent microscope (Zeiss Axio Imager. M1) and photographed by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) using a 20 \times objective. Photographs acquired with an image analysis program AxioVision (Zeiss Imaging Systems) and consisted of single c-Fos staining, single ER α or SST staining as well as merged images (c-Fos/ER α or c-Fos/SST) to produce a spectral combination of green (fluorescein) and red (rhodamine) that resulted in yellow-marked dual labelled cells. The areas examined were (as defined by Welento et al. 1969, and presented diagrammatically in Fergani et al. 2014): the VMN (4 photographs per section from random fields within each nucleus, 2 sections per ewe), ARC (3 photographs per section, 3 sections per ewe, which consisted sections from the rostral, middle and caudal divisions of the nucleus) and, for ER α only, mPOA (at the level of the OVLT: 2 photographs per section, 3 sections per ewe).

All photographs were imported into Image J version 1.42q, where counts were performed using the cell count plug-in. Initial counts were carried out on the merged image and c-Fos and ER α or SST co-localisation was confirmed using side-by-side images of the individual c-Fos and ER α or SST micrographs and visually identifying cells that contained both c-Fos label (in the nucleus) and ER α or SST label (in the cytoplasm) with respect to microscopic tissue landmarks. The mean total number and percentage of single- or dual-labelled cells was

summed from the photographs of each area/section and then averaged for each ewe and compared with GLM ANOVA, followed, where appropriate, by Tukey's multiple comparisons *post hoc* test. Mean (\pm SEM), as presented in the Results and Fig. 2 was calculated by averaging values for each group.

During data analysis, it became clear that there was a split response in the insulin group regarding the percentage of ER α or SST cells that co-expressed c-Fos. Therefore, this group was separated into two subgroups referred to hereafter as insulin-responders (IR) or insulin-non-responders [INR; verified previously in Fergani et al. (2014) as those ewes with or without c-Fos activation in the paraventricular nucleus, respectively]. As this division reduced the group size to n=2 per group, statistical analysis was not undertaken, but the data are presented for information; data were combined for analysis when responses for the insulin sub-groups did not appear different as estimated by eye.

Results

None of the animals showed any signs of illness after insulin administration. One animal from the insulin group exhibited oestrus and was mounted by a ram within 28 h after progesterone withdrawal (i.e., before the predetermined time of treatment). The data from this ewe were excluded from further analyses.

Plasma hormone concentrations

None of the animals began an LH surge during the study. Peripheral cortisol, progesterone and oestradiol profiles for the remaining ewes have been previously presented in detail (Fergani et al. 2014). Briefly, cortisol concentrations in all insulin-treated animals were elevated 2 h after insulin administration compared to controls (from 9.5 ± 0.7 to 70.4 ± 5.8 ng/ml; $p < 0.001$). Control and both insulin sub-groups had similar concentrations of progesterone before and after treatment ($p > 0.05$), whereas 2 h after insulin, oestradiol concentrations were lower in all insulin-treated animals compared to controls (from 9.5 ± 0.8 to 4.1 ± 0.4 pg/ml; $p < 0.05$).

c-Fos and ER α or SST co-expression in the hypothalamus

ARC

The number of c-Fos positive cells increased at 31 h in control and all insulin-treated animals compared to 0 h ($p < 0.05$; Table 1). The number of cells containing ER α or SST did not differ between time points in the follicular phase or after treatment (Table 1).

Photomicrographs of sections from the ARC labelled for ER α and/or c-Fos are exemplified in Fig 1. The percentage of ER α cells that co-expressed c-Fos in controls increased at 31 h ($p < 0.001$; compared to 0 h, Fig. 2A) but the percentage in insulin-treated animals did not differ from controls at 31 h (Fig. 2A). At 31 h after PW (i.e., 3 h after insulin administration), the percentage of SST cells that co-expressed c-Fos in the ARC was greater in insulin-treated animals compared to both control groups ($p < 0.05$; Fig. 2B).

VMN

The number of ER α cells was not different between 0 h and 31 h after PW in control animals (Table 1). However, all insulin-treated animals had more ER α cells compared to both 0 h and 31 h control groups ($p < 0.05$; Table 1). The number of SST cells did not differ between time points in the follicular phase or after treatment (Table 1).

Percentages of ER α cells in the 31 h control group varied considerably between animals and were not statistically different from the 0 h control group. However, at 31 h after PW (i.e., 3 h after insulin), there was circumstantial evidence to indicate that there was a marked increase in the percentage of ER α neurones that co-expressed c-Fos in the two insulin-responders, but not in the two insulin non-responders (Fig 2C). Similarly, at 31 h after PW (i.e., 3 h after insulin), there was circumstantial evidence to indicate that the percentage of SST cells that co-expressed c-Fos in the VMN increased only in the two insulin-responders (Fig. 2D).

mPOA

The number of c-Fos positive cells increased in all insulin-treated animals, compared to 0 h and 31 h controls ($p < 0.05$; Table 1). The number of ER α cells did not differ between time points in the follicular phase or after treatment (Table 1).

There was an increase in ER α co-expression with c-Fos in the mPOA, with the 31 h control group having a higher percentage of activated ER α cells compared to 0 h ($p < 0.01$; Fig. 2E). However, at 31 h after PW (i.e., 3 h after insulin), there was circumstantial evidence to indicate that there was a markedly lower percentage of ER α neurones that co-expressed c-

Fos in the insulin-responders (compared with 31 h controls and 31 h insulin non-responders; Fig. 2E).

Discussion

Our understanding of inter-relationships between hypothalamic regions during the late follicular phase has been enhanced by comparing normal c-Fos activation with that after IIH. A number of ER α cells were activated at the onset of the follicular phase in the ARC and mPOA, and this activation increased during the late follicular phase and prior to the LH surge. However, IIH given a few hours prior to the expected LH surge onset disrupted this pattern in a brain region-specific manner. In the ARC, activation of ER α neurones 3 h after IIH did not differ from controls, although there was marked increased activation of SST cells in all insulin-treated ewes (part of the glucose-sensing system). In the VMN, increased c-Fos activation in ER α and SST cells appeared to occur only in ewes with an activated PVN (measured by the presence of c-Fos; i.e., insulin-responders; Fergani et al. 2014). In the mPOA, there was circumstantial evidence to indicate that activation of ER α cells was suppressed in insulin responders. Given the important role the mPOA has in the GnRH surge mechanism (Hoffman et al. 2011; Merkley et al. 2012; Fergani et al. 2013), these observations support our hypothesis that insulin-induced activation of inhibitory SST neurones in the VMN prevents ER α -cell activation in the mPOA and leads to delay or suppression of the GnRH/LH surge.

Hypoglycaemia is induced within 3 h after insulin administration and is considered to act centrally, leading to GnRH/LH pulse inhibition and, hence, decreased peripheral oestradiol concentrations and disruption of the surge mechanism (Dobson and Smith 2000; Smith et al. 2003). There is evidence for an effect of insulin inhibiting steroidogenesis directly at ovarian level (Downing et al. 1999). However, the GnRH pulse and surge generator is particularly sensitive to reduced glucose concentrations (Medina et al. 1998). Transcriptional activation in the ARC increased in all insulin-treated animals probably because this area plays a pivotal role in glucose-sensing and energy balance (Cone et al. 2001; Routh 2003). Therefore, it is a prime candidate for linking energy status with reproduction. Within the ARC, it is clear that cells containing pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) are involved in metabolism regulation (Cone et al. 2001; Backholer et al. 2010; Myers and Olson 2012). Furthermore, recent evidence suggests that AgRP and POMC cells are able to directly influence GnRH neurone excitability in mice (Roa and Herbison 2012). Activation of these

cells may constitute a potential pathway by which IIH exerts effects on GnRH cells to inhibit production and/or release of GnRH. Our results suggest that SST-cells in the VMN may also be involved in this inhibition, as these cells were also activated 3 h after IIH in insulin responders.

Recent findings in dogs report that an intracerebroventricular injection of SST is able to increase glucose and decrease insulin levels in the periphery (Yavropoulou et al. 2014), clearly implicating this neuropeptide in metabolic regulation. In addition, SST has been strongly implicated in reproductive processes. Infusions of SST inhibit the LH surge when administered centrally and SST receptors (SST-R2) are co-localized within ovine GnRH neurones in the mPOA (Van Vugt et al. 2004; Robinson et al. 2010). Combining these independent observations provides substantial evidence for a pathway involving SST cells in the hypothalamus that, under oestradiol and potentially energy status-control, directly affect GnRH secretion.

In the mPOA, SST fibres have been identified in close apposition to GnRH neurones; whether direct contact occurs with GnRH fibres and/or cell bodies is unresolved. In mice and sheep, 50-80% GnRH neurones in the mPOA are in close apposition to at least one SST fibre or cell body (Goubillon et al. 2002; Bhattarai et al. 2010; Robinson et al. 2010), although less than 10% were identified with contacts in rats (Koyama et al. 2012). In vitro, SST suppresses GnRH neuronal firing in approximately 55-80% of GnRH neurones via SST-R2 located on the dendritic membrane, probably through volume transmission rather than synaptic transmission (Bhattarai et al. 2010; Koyama et al. 2012). Although these studies clearly demonstrate that SST is effective in suppressing the electrical activity of many GnRH neurones, some GnRH neurones are not responsive, indicating a degree of heterogeneity within the GnRH neurone population. This may be explained by variation in SST-R2 expression in distinct populations of GnRH neurones, or SST may act in combination with other inhibitory neurones, which need investigating in the future to understand the mechanisms regulating the activity of GnRH neurones.

Retrograde labelling has identified strong reciprocal connections between the VMN and ARC as well as significant input to both the ARC and VMN from the PVN (Qi et al. 2008). There is a subset of ER α neurones that project from the VMN to the ARC (Jansen et al. 1997) and another set that project from the ARC to the VMN (Elmqvist 2001) but their precise role in control of GnRH secretion has yet to be determined. It would be instructive to identify the full phenotype of cells in the ARC that project to the VMN, and vice versa. Some of the cells projecting from ARC to VMN are immuno-positive for NPY, galanin, adrenocorticotropin (a

marker for beta-endorphin) or tyrosine hydroxylase (a marker for dopamine) but their steroid receptor status is unknown (Qi et al. 2008; Whitelaw et al. 2012). Anterograde labelling also revealed projections from the ARC and VMN to the POA (Qi et al. 2008), a pathway enabling delivery of information to GnRH cells in the POA; but again, full phenotyping of these cells is required. Our data circumstantial data indicate that the pathway involving SST cells in the ARC/VMN and their projections to GnRH cells located in the mPOA merit further investigation.

IIH activates the hypothalamic-pituitary-adrenal axis leading to a consequent release of corticotropin releasing factor (CRF) from the PVN, adrenocorticotrophic hormone (ACTH) from the pituitary and cortisol from the adrenal gland (Dobson and Smith 2000). The possible activation of ER α and SST in the ARC/VMN and decreased activation of ER α in the mPOA could also have occurred via/or in addition to the activation of the stress pathway. However, we have recently shown that cells containing CRF receptor type 2 are not activated after IIH and alternative signaling may be involved (Fergani et al. 2014). Plasma cortisol concentrations increase within 3 h after IIH, whether the LH surge is delayed or not (Fergani et al. 2012; Fergani et al. 2013). This indicates that cortisol alone is not responsible for LH surge disruption after insulin. In support of this, the insulin-induced LH surge delay is not reversed by the progestin/glucocorticoid receptor antagonist RU486 (Dobson and Smith 2000). Interestingly, Wagenmaker et al. (2009) report similar findings after the application of a layered psychosocial stress paradigm, i.e., that stressor appears to have a central effect by attenuating GnRH pulses but this is not reversed by RU486, indicating that cortisol is not a mediator. It is possible that IIH and psychosocial stress are not very intense stressors (low adrenal stimulation) and, therefore, cortisol production is not sufficient to have a hypothalamic effect. Indeed, it required high-dose infusions of cortisol to disrupt the positive feedback effect of oestradiol and block the LH surge (Pierce et al. 2009; Wagenmaker et al. 2009). However, it is accepted that ~ 70% of ER α cells in the mPOA and ARC do co-express glucocorticoid receptors type II (Dufourny and Skinner 2002).

In the present study, there was circumstantial evidence to indicate that there was a split response 3 h after insulin treatment with two out of four ewes having a marked increase in the percentage of activated ER α neurones in the VMN, and a concurrent decrease in the mPOA (insulin-responders); whereas, the remaining two ewes appeared not to differ from controls (insulin non-responders). We have previously shown that this split response does not involve insulin-resistance (Fergani et al. 2012). Clearly, our present preliminary data need to be re-enforced by studying responses in a greater number of animals, but an equivalent divergence

was observed in our previous studies when 10 out of 20 animals treated with insulin did not have a delay in the LH surge (Fergani et al. 2012) and the same animals do not display intense transcriptional activation in the PVN and VMN (insulin non-responders; Fergani et al. 2014). The reason for this divergence is not known as the only observed peripheral hormonal difference between the two groups of animals was a subtle increase in plasma progesterone (Fergani et al. 2012). The location and phenotype of cells with progesterone receptors in insulin-treated ewes has not yet been determined. In contrast, the percentage of activated ER α neurones in the ARC increased in both insulin sub-groups 3 h after treatment. This concurs with our recent findings that acute IIH in the late follicular phase immediately increases the number of activated kisspeptin cells in the ARC (Fergani et al. 2014), 98% of which co-express ER α (Franceschini et al. 2006). Therefore, the increased percentage of activated ER α neurones observed in the present study may be kisspeptin cells, at least in part. Interestingly, plasma oestradiol concentrations decrease 3 h after the administration of insulin (Fergani et al. 2012; Fergani et al. 2014). However, in the present study this was not paralleled by a decrease in the percentage of activated ER α neurones in the ARC. Indeed, there appeared to be a simultaneous increase in activated ER α neurones in insulin responders in the VMN but a decrease in the mPOA.

Responses in the present study can be directly compared to those after administration of an immuno-modulatory stressor, endotoxic lipopolysaccharide from *E coli* (LPS) as we studied all animals and tissues simultaneously (Fergani et al. 2014; Sheldon et al. 2014). In brief, contrary to IIH: in the ARC, LPS decreased ER α neurone activation but had no effect on activation of SST neurones (a glucose-sensing function); in the VMN, LPS had no effect on ER α neurone activation but increased SST activation (hence, possibly interfering with the GnRH/LH surge); and, in the mPOA, ER α activation was suppressed in LPS (again, possibly interfering with the GnRH/LH surge). Making such comparisons emphasises the need to study a variety of stressors that delay/suppress the GnRH/LH surge in order to determine the core mechanism that affects the GnRH/LH surge without being side-tracked by stressor-specific responses.

In conclusion, we have shown that the normal c-Fos activation patterns in the ARC, and possibly the VMN and mPOA, are disturbed by acute IIH in the late follicular phase. Insulin stimulates SST activation in the ARC of all ewes as part of the glucose-sensing mechanism but ER α activation is unaffected by insulin in this region. We propose that disruption of the GnRH/LH surge would have only occurred in those insulin-treated ewes with an activated PVN (insulin responders). Only in these latter animals did SST activation in the VMN appear

to increase along with possible decreased ER α activation in the mPOA: similar patterns occurred after the stressor LPS indicating a common pathway (Fergani et al. 2015).

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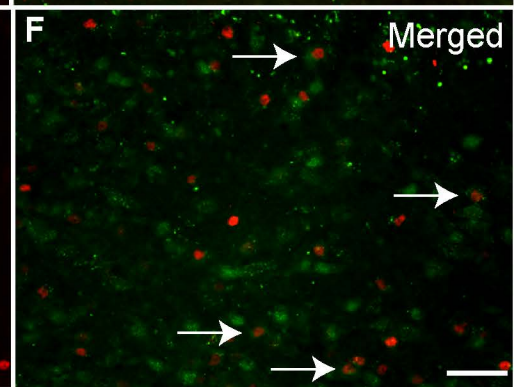
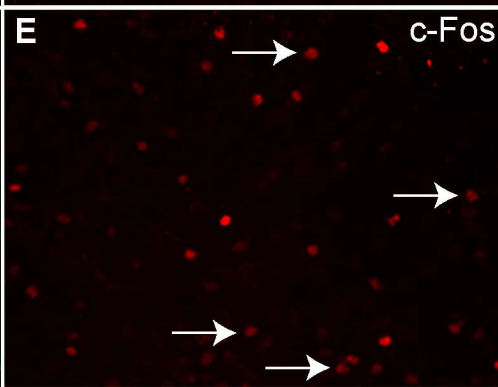
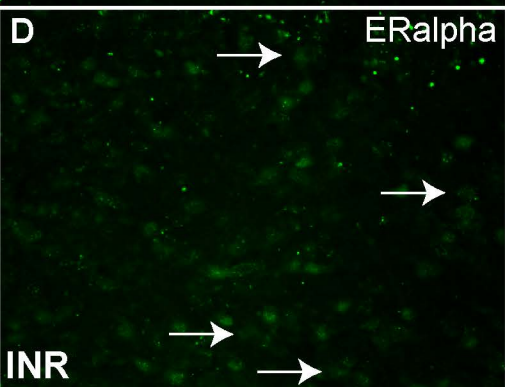
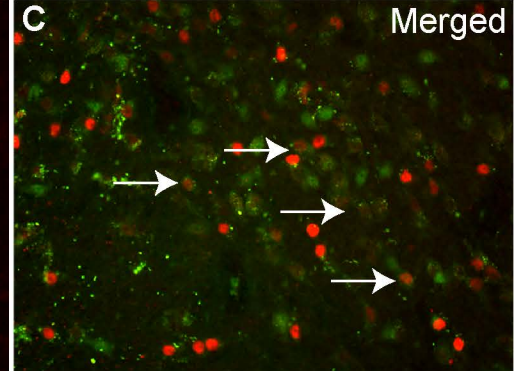
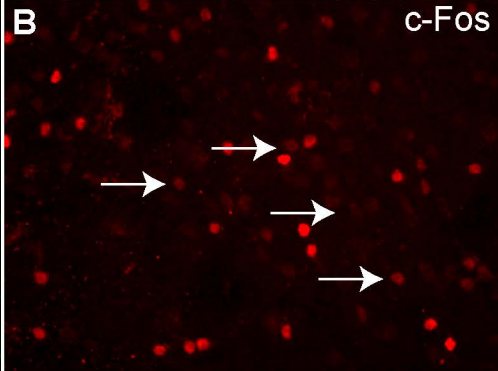
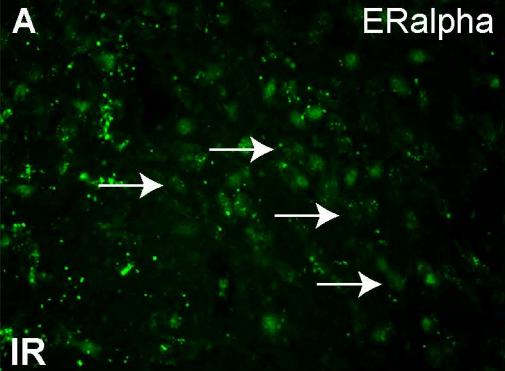
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Fig. 1. Example sets photomicrographs from the ARC that were dual-labelled for ER α cells (A,D) and their co-expression with c-Fos (B,E) 3 h after insulin treatment during the follicular phase in an insulin-responder (IR; A, B, C) and an insulin-non-responder (INR; D,E,F). Panels on the right (C, F) are computer-generated merged images of the left panels illustrating co-expression of ER α and c-Fos. Examples of double labelled cells are marked through the panels with arrows. *Scale bars* = 50 μ m.

Fig 2. Mean (\pm SEM) % of ER α or SST cells that co-express c-Fos (%ER α /c-Fos and %SST/c-Fos, respectively) in the ARC, VMN and mPOA in the follicular phase: control (C) ewes at 0 h and 31 h (n=5 and 6 per group; white bars) and after insulin at 31 h [insulin-responders (IR) n=2; black bars and insulin non-responders (INR), n=2; grey bars]. Due to the split response in the mPOA and VMN after insulin treatment, statistical analysis was not carried out and the data are presented only for information. However, in the ARC, no split responses were observed and, therefore, statistical analysis was carried out with both groups combined (n=4). Treatment with insulin was at 28h after PW. Within each panel, differences between percentages are indicated by different letters on top of each bar ($p < 0.05$).

Table 1 Mean number (\pm SEM) of cells containing c-Fos, oestradiol receptor α (ER α) or somatostatin (SST) per section in the arcuate nucleus (ARC), ventromedial nucleus (VMN) and medial preoptic area (mPOA) of the hypothalamus.



Group	Region							
	Number of c-Fos positive cells			Number of ER α positive cells			Number of SST positive cells	
	ARC	VMN	mPOA	ARC	VMN	mPOA	ARC	VMN
0 h control (n=5)	86.1 \pm 19.4	65.2 \pm 4.0	45.8 \pm 6.6	52.3 \pm 26.9	38.0 \pm 8.3	15.2 \pm 4.1	48.9 \pm 15.1	29.8 \pm 9.9
31 h control (n=6)	171.5 \pm 26.5 ^a	96.0 \pm 28.2	79.0 \pm 18.1	96.6 \pm 21.1	49.6 \pm 14.4	39.5 \pm 13.0	36.5 \pm 10.3	22.3 \pm 6.0
31h IR* (n=2)	226.5 \pm 12.0 ^a	199.0 \pm 21.0	90.0 \pm 18.5 ^a	79.2 \pm 2.2	139.0 \pm 24.0 ^{ab}	32.5 \pm 13.5	36.0 \pm 4.8	54.8 \pm 14.8
31h INR* (n=2)	259.3 \pm 47.3 ^a	75.5 \pm 10.5	143.8 \pm 10.8 ^a	156.8 \pm 77.3	72.0 \pm 9.0 ^{ab}	81.5 \pm 22.5	29.2 \pm 9.8	44.5 \pm 19.5

*Statistics were carried out with all insulin treated animals (n=4). P<0.05 compared to ^a0h or ^b31h

