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

4
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24 **Keywords:** c-Fos, oestradiol receptor alpha, somatostatin, stress, oestrus behaviour, LH
25 surge, insulin, ewes.

26

27 **Abstract**

28

29 The aim of this study was to investigate how acute insulin-induced hypoglycaemia (IIH)
30 alters the activity of cells containing oestradiol receptor α (ER α) or somatostatin (SST) in the
31 arcuate nucleus (ARC) and ventromedial nucleus (VMN), and ER α cells in the medial
32 preoptic area (mPOA) of intact ewes. Follicular phases were synchronised with progesterone
33 vaginal pessaries. Control animals were killed at 0h or 31h (n=5 and 6, respectively) after
34 progesterone withdrawal (PW; time zero). At 28h, 5 other animals received insulin (INS; 4
35 iu/kg) and were subsequently killed at 31h. Hypothalamic sections were immuno-stained for
36 ER α or SST each with c-Fos, a marker of neuronal transcriptional activation. Insulin did not
37 alter the percentage of activated ER α cells in the ARC, however, there was circumstantial
38 evidence to indicate that two insulin-treated animals (INS responders, usually with
39 suppressed LH surge) had an increase in the VMN (from 32 to 78%) and a decrease in the
40 mPOA (from 40 to 12%) compared to no increase the two INS non-responders (usually with
41 LH surge). The percentage of activated SST cells in the ARC was greater in all four insulin-
42 treated animals (from 10 to 60%), whereas there was circumstantial evidence to indicate that
43 activated SST cells in the VMN increased only in the two insulin-responders (from 10 to
44 70%). From these results, we suggest that IIH stimulates SST activation in the ARC as part of
45 the glucose-sensing mechanism but ER α activation is unaffected in this region. We present
46 circumstantial evidence to support a hypothesis that disruption of the GnRH/LH surge may
47 occur in insulin responders via a mechanism that involves, at least in part, SST cell activation
48 in the VMN along with decreased ER α cell activation in the mPOA.

49

50 **Introduction**

51

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

59

60 The action of oestradiol upon the mammalian brain occurs mainly through classical
61 transcriptional action, namely oestrogen receptor alpha (ER α) signalling (McEwen et al.
62 2012, Cheong et al. 2014). However, steroid hormone signals do not impinge directly on
63 GnRH cells as these cells do not possess progesterone receptors (PR) or ER α (Shivers et al.
64 1983; Skinner et al. 2001). Some GnRH neurones express, ER β (Hrabovszky et al. 2001)
65 although it is unlikely that ER β plays a major role in the feedback regulation of GnRH/LH
66 secretion, because ER β knock-out mice have normal fertility (Lubahn et al. 1993; Cheong et
67 al. 2014).

68

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

76

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

84 term oestradiol treatment in progesterone-primed ovariectomised ewes increases SST
85 activation in the VMN approximately 10 h before the anticipated onset of an LH surge (Pillon
86 et al. 2004; Robinson et al. 2010). Conversely, in rats, SST is one of the most potent
87 inhibitors of electrical excitability of GnRH neurones identified thus far (Bhattarai et al.
88 2010) and SST inhibits the LH surge when administered centrally (Van Vugt et al. 2004).
89 Interestingly, recent evidence suggests that hypothalamic SST is also implicated in glucose
90 metabolism by initiating a cascade of events that lead to a peripheral increase in glucose and
91 decrease in insulin (Yavropoulou et al. 2014). It is, therefore, possible that SST cells are
92 activated during insulin-induced disruption of the LH surge and provide an important link
93 between metabolism and reproduction.

94

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

100

101 **Materials and Methods**

102

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

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

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

120

121 *Tissue collection*

122

123 Euthanasia was carried out with sodium pentobarbitone containing 25,000 IU heparin; full
124 details of fixation (Zamboni; picric acid, paraformaldehyde and sucrose) and preservation (at
125 -80°C) of tissues are given in (Fergani et al. 2014). Free-floating ($40\ \mu\text{m}$) coronal sections
126 were stored in cryoprotectant solution and stored at -20°C until processed for
127 immunohistochemistry.

128

129 *c-Fos and ER α or SST dual-label immunofluorescence*

130

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

135

136 Details of the c-Fos methodology (antibody AB-5, PC38, Calbiochem, Cambridge, MA,
137 USA; at a dilution of 1:5000) have already been described (Fergani et al. 2013). This was
138 modified in the present study by co-incubating the polyclonal rabbit anti c-Fos antibody for
139 72 h with a monoclonal mouse anti-ER α antibody (ID5, M7047, Dako, Carpinteria, CA,
140 USA) at a dilution of 1:50. After incubation, sections were washed thoroughly and incubated
141 with a mixture of donkey anti-rabbit Cy3 (711-165-152, Jackson Immunoresearch, West
142 Grove, PA) and donkey anti-mouse DyLight 488 (715-485-151, Jackson Immunoresearch,
143 West Grove, PA) both diluted at 1:500 for 2 h. Thereafter, sections were washed with PBS
144 followed by a final wash with double-distilled water, mounted on chrome alum gelatine
145 coated slides and cover-slipped with Vectashield anti-fading mounting medium (Vector
146 Laboratories Ltd, UK, H-1000).

147

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

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

154

155 The c-Fos (Ghuman et al. 2011), ER α (Dufourny and Skinner 2002) and SST (Robinson et al.
156 2010) antibodies have been validated for the use in ovine neural tissue. In addition, negative
157 controls that omitted one of the primary antibodies completely eliminated the appropriate
158 fluorescence without noticeably affecting the intensity of the other fluorescent probe.

159

160 *Data analysis*

161

162 Hormone and immunohistochemistry data were analysed with Minitab® 15 statistical
163 package (MINITAB Inc, Pennsylvania, USA). Statistical significance was accepted when $p <$
164 0.05.

165

166 Histological sections were examined under an epi-fluorescent microscope (Zeiss Axio
167 Imager. M1) and photographed by digital microphotography (Hamamatsu ORCA I-ER digital
168 camera, Hamamatsu Photonics, Welwyn Garden City, Herts) using a 20 \times objective.

169 Photographs acquired with an image analysis program AxioVision (Zeiss Imaging Systems)
170 and consisted of single c-Fos staining, single ER α or SST staining as well as merged images
171 (c-Fos/ER α or c-Fos/SST) to produce a spectral combination of green (fluorescein) and red
172 (rhodamine) that resulted in yellow-marked dual labelled cells. The areas examined were (as
173 defined by Welento et al. 1969, and presented diagrammatically in Fergani et al. 2014): the
174 VMN (4 photographs per section from random fields within each nucleus, 2 sections per
175 ewe), ARC (3 photographs per section, 3 sections per ewe, which consisted sections from the
176 rostral, middle and caudal divisions of the nucleus) and, for ER α only, mPOA (at the level of
177 the OVLT: 2 photographs per section, 3 sections per ewe).

178

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

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

189

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

198

199 **Results**

200

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

205

206 *Plasma hormone concentrations*

207

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

216

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

218

219 *ARC*

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

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

229

230 *VMN*

231 The number of ER α cells was not different between 0 h and 31 h after PW in control animals
232 (Table 1). However, all insulin-treated animals had more ER α cells compared to both 0 h and
233 31 h control groups ($p < 0.05$; Table 1). The number of SST cells did not differ between time
234 points in the follicular phase or after treatment (Table 1).

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

243

244 *mPOA*

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

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

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

254

255 **Discussion**

256

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

273 Hypoglycaemia is induced within 3 h after insulin administration and is considered to act
274 centrally, leading to GnRH/LH pulse inhibition and, hence, decreased peripheral oestradiol
275 concentrations and disruption of the surge mechanism (Dobson and Smith 2000; Smith et al.
276 2003). There is evidence for an effect of insulin inhibiting steroidogenesis directly at ovarian
277 level (Downing et al. 1999). However, the GnRH pulse and surge generator is particularly
278 sensitive to reduced glucose concentrations (Medina et al. 1998). Transcriptional activation in
279 the ARC increased in all insulin-treated animals probably because this area plays a pivotal
280 role in glucose-sensing and energy balance (Cone et al. 2001; Routh 2003). Therefore, it is a
281 prime candidate for linking energy status with reproduction. Within the ARC, it is clear that
282 cells containing pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) are
283 involved in metabolism regulation (Cone et al. 2001; Backholer et al. 2010; Myers and Olson
284 2012). Furthermore, recent evidence suggests that AgRP and POMC cells are able to directly
285 influence GnRH neurone excitability in mice (Roa and Herbison 2012). Activation of these

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

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

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

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

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

327 IIH activates the hypothalamic-pituitary-adrenal axis leading to a consequent release of
328 corticotropin releasing factor (CRF) from the PVN, adrenocorticotrophic hormone (ACTH)
329 from the pituitary and cortisol from the adrenal gland (Dobson and Smith 2000). The possible
330 activation of ER α and SST in the ARC/VMN and decreased activation of ER α in the mPOA
331 could also have occurred via/or in addition to the activation of the stress pathway. However,
332 we have recently shown that cells containing CRF receptor type 2 are not activated after IIH
333 and alternative signaling may be involved (Fergani et al. 2014). Plasma cortisol
334 concentrations increase within 3 h after IIH, whether the LH surge is delayed or not (Fergani
335 et al. 2012; Fergani et al. 2013). This indicates that cortisol alone is not responsible for LH
336 surge disruption after insulin. In support of this, the insulin-induced LH surge delay is not
337 reversed by the progestin/glucocorticoid receptor antagonist RU486 (Dobson and Smith 2000).
338 Interestingly, Wagenmaker et al. (2009) report similar findings after the application of a
339 layered psychosocial stress paradigm, i.e., that stressor appears to have a central effect by
340 attenuating GnRH pulses but this is not reversed by RU486, indicating that cortisol is not a
341 mediator. It is possible that IIH and psychosocial stress are not very intense stressors (low
342 adrenal stimulation) and, therefore, cortisol production is not sufficient to have a
343 hypothalamic effect. Indeed, it required high-dose infusions of cortisol to disrupt the positive
344 feedback effect of oestradiol and block the LH surge (Pierce et al. 2009; Wagenmaker et al.
345 2009). However, it is accepted that ~ 70% of ER α cells in the mPOA and ARC do co-express
346 glucocorticoid receptors type II (Dufourny and Skinner 2002).

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

354 was observed in our previous studies when 10 out of 20 animals treated with insulin did not
355 have a delay in the LH surge (Fergani et al. 2012) and the same animals do not display
356 intense transcriptional activation in the PVN and VMN (insulin non-responders; Fergani et al.
357 2014). The reason for this divergence is not known as the only observed peripheral hormonal
358 difference between the two groups of animals was a subtle increase in plasma progesterone
359 (Fergani et al. 2012). The location and phenotype of cells with progesterone receptors in
360 insulin-treated ewes has not yet been determined. In contrast, the percentage of activated ER α
361 neurones in the ARC increased in both insulin sub-groups 3 h after treatment. This concurs
362 with our recent findings that acute IIH in the late follicular phase immediately increases the
363 number of activated kisspeptin cells in the ARC (Fergani et al. 2014), 98% of which co-
364 express ER α (Franceschini et al. 2006). Therefore, the increased percentage of activated ER α
365 neurones observed in the present study may be kisspeptin cells, at least in part. Interestingly,
366 plasma oestradiol concentrations decrease 3 h after the administration of insulin (Fergani et
367 al. 2012; Fergani et al. 2014). However, in the present study this was not paralleled by a
368 decrease in the percentage of activated ER α neurones in the ARC. Indeed, there appeared to
369 be a simultaneous increase in activated ER α neurones in insulin responders in the VMN but a
370 decrease in the mPOA.

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

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

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

390

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396

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520

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

527

528 **Fig 2.** Mean (\pm SEM) % of ER α or SST cells that co-express c-Fos (%ER α /c-Fos and
529 %SST/c-Fos, respectively) in the ARC, VMN and mPOA in the follicular phase: control (C)
530 ewes at 0 h and 31 h (n=5 and 6 per group; white bars) and after insulin at 31 h [insulin-
531 responders (IR) n=2; black bars and insulin non-responders (INR), n=2; grey bars]. Due to
532 the split response in the mPOA and VMN after insulin treatment, statistical analysis was not
533 carried out and the data are presented only for information. However, in the ARC, no split
534 responses were observed and, therefore, statistical analysis was carried out with both groups
535 combined (n=4). Treatment with insulin was at 28h after PW. Within each panel, differences
536 between percentages are indicated by different letters on top of each bar ($p < 0.05$).

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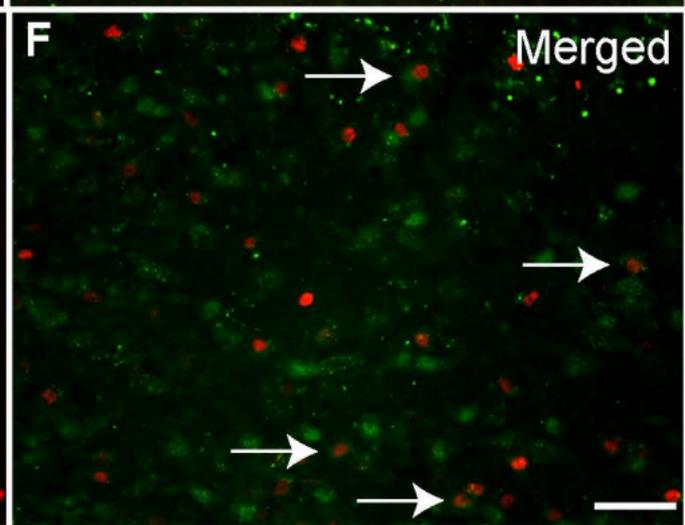
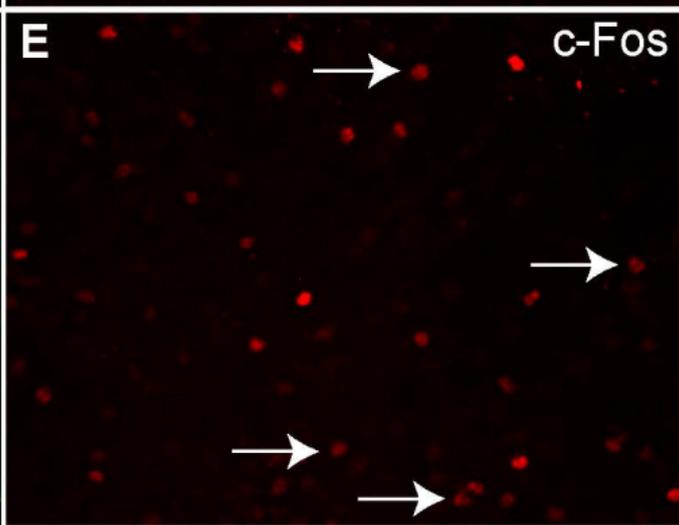
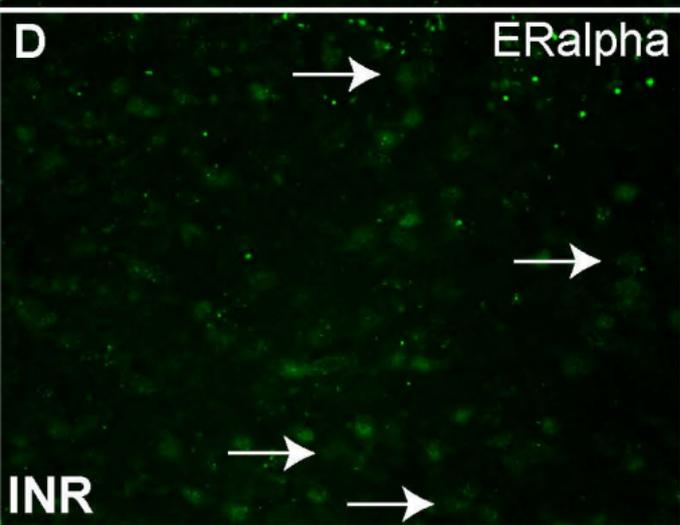
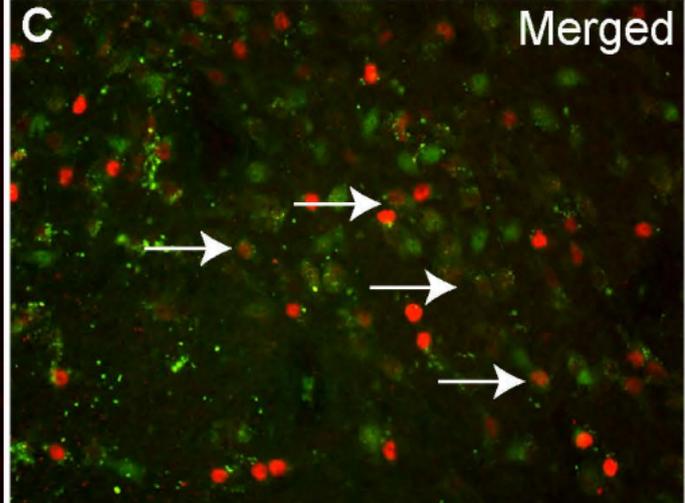
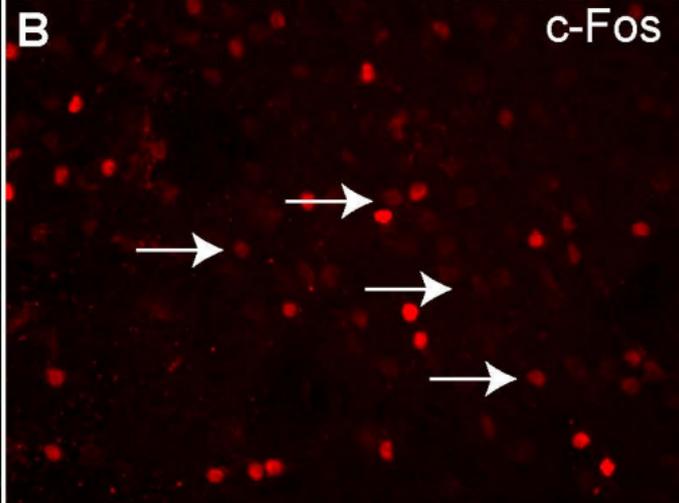
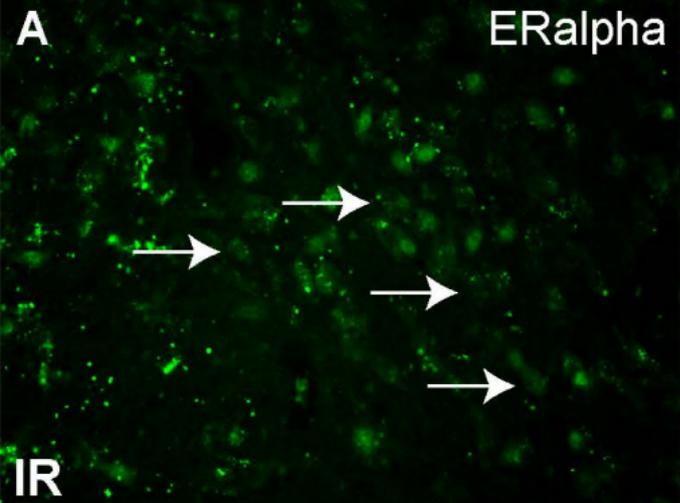
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540 **Table 1** Mean number (\pm SEM) of cells containing c-Fos, oestradiol receptor α (ER α) or
541 somatostatin (SST) per section in the arcuate nucleus (ARC), ventromedial nucleus (VMN)
542 and medial preoptic area (mPOA) of the hypothalamus.

543

544



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

