The arcuate nucleus kisspeptin response to increased nutrition in rams

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Abstract

Rams respond to acute nutritional supplementation by increasing the frequency of GnRH pulses. Kisspeptin neurons may mediate the effect of environmental cues on GnRH secretion, so we tested whether the ram response to nutrition involves activation of kisspeptin neurons in the arcuate nucleus (ARC) – KNDy neurons. Rams were given extra lupin grain with their normal ration. Blood was sampled for prior to feeding, and continued until animals were killed for collection of brain tissue at 2 or 11 h post-supplementation. In supplemented rams, LH pulse frequency increased after feeding, whereas control animals showed no change. Within the caudal-ARC, there were more kisspeptin neurons in supplemented rams than in controls and a higher proportion of kisspeptin cells co-expressed Fos, regardless of time of euthanasia. There were more Fos cells in the mid-ARC and mid dorsomedial hypothalamus (DMH) of the supplemented rams compared to control rams. No effect of nutrition was found on kisspeptin expression in the rostral- or mid-ARC, or on GnRH expression in the pre-optic area (POA). Kisspeptin neurons in the caudal-ARC appear to mediate the increase in GnRH/LH production due to acute nutritional supplementation, supporting the hypothesised role of the KNDy neurons as the pulse generator for GnRH.

Introduction

Successful reproduction relies on the pulsatile secretion of gonadotrophin releasing hormone (GnRH), with changes in the frequency of these pulses switching reproduction between active and inactive states. The secretion of GnRH pulses is regulated by an integration of neuronal inputs reflecting environmental factors, such as photoperiod and nutrient availability, and endogenous factors, such as sex steroid feedback (Tjondronegoro *et al.* 1996; Blache *et al.* 1997). In many species, the regulation of GnRH secretion in this way allows reproduction to be timed advantageously, for example switching on reproduction when sufficient nutrition is available to support the energy demands of reproduction and this response is more prominent in females where the investment in reproduction is high. Nevertheless, the male sheep responds to an acute increase in nutrition by increasing the frequency of GnRH pulses within 6 hours of feeding (Zhang *et al.* 2004; Zhang *et al.* 2005), leading to an increase in testosterone and sperm production (Blache *et al.* 2002; Guan and Martin 2017). However, the neuronal link between hypothalamic-pituitary-gonadal (HPG) activity and nutritional cues is not fully understood. It most likely involves insulin and leptin, two metabolic hormones that are known to affect reproduction (Blache *et al.* 2006). However, GnRH neurons lack receptors for both insulin and leptin, indicating that if the link exists, it must be at least “one step back” from the GnRH neurons (Clarke 2011).

Kisspeptin is critically involved in HPG regulation, as kisspeptin administration stimulates GnRH activity, and kisspeptin antagonists inhibit it (Messenger *et al.* 2005; Roseweir *et al.* 2009). In sheep, kisspeptin neurons are found in the arcuate nucleus (ARC) and preoptic area (POA) (Smith and Clarke 2007) and those in the former are known to co-express neurokinin B (NKB) and dynorphin (Dyn) (Goodman *et al.* 2007) with their requisite receptors (Navarro *et al.* 2009; Amstalden *et al.* 2010; Weems *et al.* 2016). These 'KNDy' neurons are proposed to act via a reciprocally interconnected network to drive GnRH pulsatility, with strong data in sheep (Lehman *et al.* 2010; Goodman *et al.* 2013; Li *et al.* 2015) and mice (Navarro *et al.* 2009; Clarkson *et al.* 2017).

Evidence further suggests that kisspeptin neurons may be the 'missing link' between HPG activity and nutritional cues. KNDy neurons are known to mediate environmental effects on reproduction – they are involved in the 'ram effect', a phenomenon where ewes show a surge in HPG activity when presented with a novel ram (De Bond *et al.* 2013; Fabre-Nys *et al.* 2017). Importantly, kisspeptin also seems to be involved in responses to nutritional signals, particularly in rodents, where several lines of evidence have been published: i) fasting decreases *Kiss* mRNA and central administration of kisspeptin reverses fasting-induced inhibition of GnRH secretion (Castellano *et al.* 2005); ii) kisspeptin neurons apposite to GnRH neurons possess insulin, leptin and ghrelin receptors (Qiu *et al.* 2011; Cravo *et al.* 2013; Frazao *et al.* 2014; Qiu *et al.* 2015); iii) leptin-deficient *ob/ob* mice have lower levels of *Kiss1* mRNA in the ARC, compared to wild-type controls, and the peripheral administration of leptin reduces this effect (Smith *et al.* 2006); iv) kisspeptin also interacts with the insulin signalling pathway in the hypothalamus, as evidenced by diabetic rats having diminished expression of *Kiss1* mRNA, an effect that can be reversed with insulin therapy (Castellano *et al.* 2006); and v) impairment of kisspeptin signalling decreases metabolism and increases obesity and glucose intolerance (Tolson *et al.* 2014). Therefore, in this study, we tested whether kisspeptin is involved in the GnRH response to an acute increase in nutrition in sexually mature male sheep. We hypothesise that the stimulatory effect of a nutritional supplement on LH pulse frequency will be accompanied by activation of the KNDy cells.

Methods

Animals and treatments

The experimental procedures were approved by the Animal Ethics Committee of the University of Western Australia, according to the recommendations of the National Health and Medical Research Council of Australia under RA/3/100/1311.

Sexually experienced male sheep (2 years old; Merino; $n = 24$) were individually penned in indoor housing at the University of Western Australia ($31^{\circ} 58^{\circ}$ S). The experiment was performed in May (southern hemisphere) and photoperiod approximated external conditions 12L: 12D with lights on at 6.30 am. Housing was kept at constant temperature ($20 \pm 2^{\circ}\text{C}$) and relative humidity ($50 \pm 2\%$). Animals were acclimatised to housing and diet for a 2 wk period prior to experimentation. Animals were fed a diet designed to maintain constant bodyweight and the daily allowance consisted of chaff, lupins and minerals, to yield 8.7 MJ of metabolisable energy/KG. Animals were fed at a consistent time each day.

Rams were allocated into 2 groups, balanced for weight: control ($n = 8$) 69.9 ± 3.74 kg BW and supplemented ($n = 16$) 70.4 ± 2.46 kg BW. Sampling was conducted over 2 days. Jugular cannulas were inserted into each animal the day prior to sampling. On each sampling day, blood was sampled every 20 min beginning 8 h prior to the regular feeding time. At feeding time animals were fed the regular ration (control group) or the regular ration plus a supplement of lupin grain (30 g/kg body weight, supplemented group). Blood sampling continued until animals were killed and brains collected, at either 2 h (control $n = 5$, supplemented $n = 7$) or 11 h post-feeding (control $n = 3$, supplemented $n = 9$).

Animals were killed by an intravenous overdose of sodium pentobarbital (Lethabarb; Virbac, Peakhurst, NSW, Australia), then heads quickly removed, and brains perfused with 2 L of 0.9% saline and 3 L of 4% paraformaldehyde (pH 7.4). Blocks of tissue containing the hypothalamus were dissected out and stored in 4% paraformaldehyde for a further 24 h (4 °C) followed by 24–48 h in 15% sucrose (4 °C). Tissue was embedded (OCT; ProSciTech, Thuringowa, QLD, Australia) and stored at -80 °C. Coronal sections of the hypothalamus were cut at 40 µm thickness on a cryostat (CM3050; Leica, Wetzlar, Germany), and stored in cryoprotective fluid (20% glycerol, 30% ethanediol in 0.1M phosphate buffer at pH 7.3) at -20 °C.

Immunohistochemistry

Kisspeptin/Fos double-label immunohistochemistry

Anatomically matched single sections representing the rostral, middle, and caudal regions of the ARC approximately 300 µm apart were chosen from each ram. Free-floating sections were washed in 0.1 M tris-buffered saline (TBS), and incubated in 1% sodium borohydride, followed by a blocking serum (0.1 M TBS, 10% normal goat serum, 0.3% Triton X-100), and then a rabbit polyclonal antibody raised against Fos protein diluted in a solution of 10% normal goat serum, 2% sodium azide and 0.3% Triton X-100 in TBS (1:500, overnight at room temperature; sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Fos was visualised with a goat anti-rabbit secondary antibody (1:400, Alexa 488, Invitrogen, San Diego, CA, USA). Sections were again incubated in blocking serum, and then a rabbit polyclonal antibody raised against ovine kisspeptin-10 (1:2000, overnight at room temperature; no. AC566) and was a gift from A. Caraty (Universite Tours, Nouzilly, France) previously validated for use in sheep tissue (Franceschini *et al.* 2006; Goodman *et al.* 2007). Kisspeptin was visualized with a goat anti-rabbit secondary antibody (1:400, Alexa 546, Invitrogen, San Diego, CA, USA). Sections were mounted onto SuperFrost slides and left to dry overnight. Sections were stained with Sudan Black (0.3% in 70% ethanol, 3 min), washed in TBS and phosphate-buffered saline and then coverslipped with an anti-fade mounting medium (Fluoromount Dako Corp., Carpinteria, CA, USA). Cells immuno-reactive (ir) for kisspeptin, Fos and both kisspeptin and Fos were counted subjectively by an experienced individual blind to the condition of the animal.

GnRH/Fos double-label immunohistochemistry

A section representing the mid-POA was chosen from each ram. Immunohistochemistry was conducted as above. The mouse monoclonal primary antibody raised against GnRH (1:1000, MAB5456, EMD Millipore Corporation, Temecula, CA, USA) was visualised with a goat anti-mouse secondary antibody (1:400, Alexa 546, Invitrogen, San Diego, CA, USA). Cells immuno-reactive for GnRH, Fos and both GnRH and Fos were counted subjectively by an experienced individual blind to the condition of the animal.

Radioimmunoassay

Plasma LH was measured in all samples in duplicate by a double-antibody radioimmunoassay (Martin *et al.* 1980) using ovine LH (NIDDK-oLH-1-4; AFP-8614B) for iodination and standards kindly supplied by A. Parlow, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA. The limit of detection was 0.06 ng/ml. Quality control samples (0.5, 1.1 and 1.9 ng/mL) were used to calculate intra-assay variation (<10% for each).

LH pulse analysis

Pulses of LH secretion were detected with MUNRO, an adaptation of the PULSAR program (Merriam and Wachter 1982), that was developed for the Apple Macintosh computer ('Munro', Zaristow Software, West Morham, Haddington, East Lothian, UK). For the present study, the G parameters were 10, 5, 3, 2, and 1 for G1 to G5, respectively. The Baxter parameters were 0.0126 (B1, the y-intercept), 0.0218 (B2, the x coefficient), and 0.0058 (B3, the x² coefficient). The other parameters of the Munro program were a smoothing window of 60 min, a nadir window of 40 min, a minimal pulse interval of 60 min, and a rise threshold of 4 S.D.

Statistical analysis

All statistical analyses were conducted in Genstat 12.1 (Twelfth edition, Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK).

LH analysis

Mean LH was compared over 8 h before vs 2 h and 8 h after feeding between control vs supplemented animals using a repeated measures ANOVA. Pulse frequency was calculated as the number of pulses per hour (over 8 h before and after feeding) and the difference in pulse frequency was analysed using a repeated measures ANOVA. The mean pulse amplitude, area and nadir were calculated for each animal before and after feeding, from data generated by the PULSAR program. Some animals did not have an LH pulse prior to feeding, so there was no 'before' data for pulse characteristics in these animals. Therefore, pulse characteristics were analysed in two ways: First, animals without a 'before' pulse were removed from analysis ($n = 5$ from the supplemented group), and the remaining animals analysed comparing before vs after feeding and control vs supplemented animals (resulting group sizes: control: $n = 3$, supplemented: $n = 4$). For pulse nadir, this was done using a repeated measures ANOVA, and day of experimentation was used as a blocking factor. However, data for amplitude and area violated normality and homogeneity of variance assumptions, and so were analysed using a Wilcoxon Matched Pairs tests to compare before vs after feeding, and Mann-Whitney U tests to compare control vs supplemented animals either before or after feeding. These data are presented as the median and 95% CI or interquartile range.

Second, all animals were analysed comparing control and supplemented animals only after feeding (control: $n = 3$, supplemented: $n = 9$). For pulse amplitude and nadir this was done using a one-way ANOVA. Day of experimentation was used as a blocking factor. Prior to analysis pulse amplitude was transformed (logarithm base e) to adhere to normality and homogeneity of variance assumptions. Pulse area violated normality and homogeneity of variance assumptions, and so was analysed using a Mann-Whitney U test.

Results from each way of testing did not yield conflicting results. Pulse amplitude, area and nadir data presented in the paper is the mean of data from all animals killed 11 h post-feeding.

Immunohistochemistry data

One animal from the supplemented treatment killed 11 h after feeding was excluded from analysis of the hypothalamic regions because the hypothalamic sections were damaged during processing and we were unable to obtain meaningful data (resulting group sizes: control 2 h: $n = 5$, supplemented 2 h: $n = 7$, control 11 h: $n = 3$, supplemented 11 h: $n = 8$). The number of kisspeptin neurons in each region of the hypothalamus and the number of GnRH neurons in the POA was analysed using a two-way ANOVA, comparing 2 h and 11 h post-feeding and control vs supplemented animals. Day of experimentation was used as a blocking factor. Data for percentage of kisspeptin neurons co-labelling for Fos and percentage of GnRH neurons co-labelling for Fos were unable to be transformed to adhere to normality and homogeneity of variance assumptions, and so were analysed using a Kruskal-Wallis one way ANOVA, comparing collection-time and supplementation separately. Overall activation of neurons as indicated by the presence of Fos was analysed in each region using a two-way ANOVA, comparing before vs after feeding and control vs supplemented animals. Day of experimentation was used as a blocking factor.

Results

LH secretion

Representative LH profiles are shown for control and feed supplemented rams (Figure 1).

Mean LH increased immediately (2 h) following supplementation ($P < 0.05$), but was unchanged over the full 11 h post-feeding period (Figure 2A).

LH pulse frequency differed with time and supplementation, with a significant interaction ($P < 0.05$). Specifically, post feeding pulse frequency increased by over 2-fold in supplemented animals compared to control animals ($P < 0.01$; Figure 2B). Pulse amplitude, nadir, and area did not change with time, supplementation, nor was there an interaction between time and supplementation (Table 1).

Presence and activation of kisspeptin and GnRH neurons

There were more kisspeptin neurons in the caudal ARC of supplemented rams than in control rams ($P < 0.05$), regardless of time of death (Table 2 and Figure 3A and B). However, there was no difference in the number of kisspeptin neurons in either the mid or rostral regions of the ARC, nor was there an effect of time of death (Table 2).

There was a higher percentage of kisspeptin neurons co-labelled for Fos in the caudal ARC of supplemented rams than in control rams ($P < 0.05$, Figure 3 C and D); however, there was no difference in the percentage of kisspeptin neurons co-labelled for Fos in either the mid or rostral regions of the ARC (Table 2). There was no difference in the number or activation of GnRH neurons in the POA between supplemented and control rams at 2 and 11 h post-feeding (Table 2 and Figure 3 E and F).

Overall neural activation after lupin supplementation

There were more neurons labelled for Fos in the mid ARC and mid dorsomedial hypothalamus of the supplemented rams compared to the control rams (both $P < 0.05$; Table 3, Figure 3 G and H). There was no effect of supplementation on Fos activation in the regions of the rostral or caudal ARC or dorsomedial hypothalamus, the paraventricular nucleus or the POA. There was no effect of kill-time nor an interaction between kill-time and supplementation on Fos activation in any region.

Fig. 1. Plasma LH profiles are shown in representative animals fed either their usual ration (Control) or supplemented with extra lupins. The timing of feeding is indicated by the shaded area. LH pulses are indicated by arrows.

Fig. 2. Mean LH (A) and LH pulse frequency (B) in animals before and after feeding usual rations (Control) or supplemented with extra lupin grain. The pre-feeding and post-feeding periods were 8 h, with an addition 2 h post feeding group included for mean LH analysis. Group numbers: Pre-feeding and 2 h Post, control $n = 8$, supplemented $n = 16$; Post-feeding, control $n = 3$, supplemented $n = 9$. Mean $\pm$ S.E. shown. * indicates $P < 0.05$ compared to control. ** indicates $P < 0.01$ compared to control.

Table 1. LH pulse characteristics in rams fed either their usual ration (n = 3) or supplemented with extra lupin grain (n = 9). Mean $\pm$ S.E. shown.

	Pre-feeding (8 h)		Post-feeding (8 h)	
	Control	Supplemented	Control	Supplemented
Amplitude (ng/mL)	0.80 $\pm$ 0.11	0.68 $\pm$ 0.30	1.40 $\pm$ 0.72	0.60 $\pm$ 0.09
Nadir (ng/mL)	0.15 $\pm$ 0.04	0.24 $\pm$ 0.07	0.16 $\pm$ 0.03	0.18 $\pm$ 0.02
Area (ng/mL)	50.4 $\pm$ 7.17	40.0 $\pm$ 16.09	56.8 $\pm$ 25.62	26.4 $\pm$ 2.56

Table 2. Presence and activation of kisspeptin neurons within the ARC and GnRH neurons within the POA in rams fed either their usual ration (n = 8) or supplemented with extra lupin grain (n = 15), 2 or 11 h after feeding. Group numbers: control 2 h: n = 5, control 11 h: n = 3, supplemented 2 h: n = 7, supplemented 11 h: n = 7 for hypothalamic regions, 8 for pre-optic area. Mean $\pm$ S.E. shown. * Indicates ($P < 0.05$) between control and supplemented animals within that brain region.

		2 h post-feeding		11 h post-feeding	
		Control	Supplemented	Control	Supplemented
Number of ARC kisspeptin neurons	Caudal	2.4 $\pm$ 1.08	7.7 $\pm$ 0.52 *	4.3 $\pm$ 2.85	6.9 $\pm$ 1.51 *
	Mid	5.8 $\pm$ 0.73	7.0 $\pm$ 1.99	6.7 $\pm$ 4.41	4.9 $\pm$ 1.22
	Rostral	5.6 $\pm$ 1.72	7.6 $\pm$ 2.19	9.3 $\pm$ 3.18	6.0 $\pm$ 2.76
% ARC kisspeptin + Fos	Caudal	0 $\pm$ 0.00	5.0 $\pm$ 2.35 *	0 $\pm$ 0.00	3.9 $\pm$ 1.97 *
	Mid	10.0 $\pm$ 6.67	3.1 $\pm$ 2.03	2.2 $\pm$ 2.22	14.4 $\pm$ 7.27
	Rostral	4.4 $\pm$ 2.72	11.9 $\pm$ 7.76	8.1 $\pm$ 4.82	8.1 $\pm$ 2.30
Number of GnRH neurons	POA	2.4 $\pm$ 1.17	3.3 $\pm$ 0.87	3.3 $\pm$ 1.76	1.4 $\pm$ 0.59
% of GnRH + Fos	POA	3.3 $\pm$ 3.33	9.5 $\pm$ 7.14	5.6 $\pm$ 5.56	13.3 $\pm$ 12.54

Table 3. Number of neurons staining for Fos in brain regions of rams fed either their usual ration or supplemented with extra lupin grain, 2 or 11 h post-feeding. Mean $\pm$ S.E. shown. Group sizes: control 2 h: n = 5, control 11 h: n = 3, supplemented 2 h: n = 7, supplemented 11 h: n = 7 for hypothalamic regions, 8 for pre-optic area. * Indicates ($P < 0.05$) between control and supplemented animals within that brain region.

		2 h post-feeding		11 h post-feeding	
		Control	Supplemented	Control	Supplemented
Arcuate nucleus	Caudal	202 $\pm$ 61.8	304 $\pm$ 5.2	132 $\pm$ 98.1	283 $\pm$ 32.9
	Mid	485 $\pm$ 140.8	732 $\pm$ 59.5 *	245 $\pm$ 133.8	660 $\pm$ 87.1 *
	Rostral	796 $\pm$ 132.3	738 $\pm$ 190.7	403 $\pm$ 150.5	592 $\pm$ 63.3
Dorsomedial hypothalamus	Caudal	177 $\pm$ 35.5	199 $\pm$ 17.6	198 $\pm$ 41.9	233 $\pm$ 27.4
	Mid	227 $\pm$ 53.3	331 $\pm$ 45.2 *	139 $\pm$ 61.1	355 $\pm$ 40.6 *
	Rostral	399 $\pm$ 59.6	430 $\pm$ 25.4	230 $\pm$ 66.4	379 $\pm$ 46.0
Paraventricular nucleus	Caudal	94 $\pm$ 9.1	119 $\pm$ 11.0	105 $\pm$ 37.2	128 $\pm$ 18.2
	Mid	109 $\pm$ 27.7	150 $\pm$ 26.3	62 $\pm$ 32.2	161 $\pm$ 30.8
	Rostral	227 $\pm$ 20.7	211 $\pm$ 28.1	146 $\pm$ 23.2	220 $\pm$ 37.5
Pre-optic area		486 $\pm$ 47.3	477 $\pm$ 40.5	413 $\pm$ 52.9	495 $\pm$ 68.2

Fig. 3. Representative photomicrographs demonstrating kisspeptin (red) and Fos (green) labelling in rams 2 h post feeding. A-C: Kisspeptin immunoreactive cells in the arcuate nucleus of a control ram (A) and rams fed lupins (B and C). Arrows indicate positive cells. D-I: Examples of caudal ARC kisspeptin neurons co-expressing Fos in control (D-F) and lupin fed (G-I) rams (note: no co-expression was seen in control). Each panel represents kisspeptin, Fos and the merged image. 3V, third ventricle. Scale bars, 100 µm.

Discussion

In this study, we detected a small but significant increase in LH pulsatility in male sheep immediately following the presentation of a supplemented diet. Paired with this change was a small but significant increase in overall number of kisspeptin neurons in the caudal ARC and the percentage of these kisspeptin neurons co-expressing Fos – indicating activation. We also show neuronal activation of additional hypothalamic neurons in the mid-ARC and dorsomedial hypothalamus. An increase in LH pulses (assumed to be the direct result of GnRH pulses) in sheep in response to supplemented feed was first demonstrated by Martin and colleagues (Martin *et al.* 1994). At this time, it was hypothesized that an interplay between nutrition and steroid negative feedback governing GnRH neurons was key to the response (Blache *et al.* 2006). We now implicate kisspeptin neurons in the caudal ARC in the promulgation of this feeding effect.

Kisspeptin neurons in the ARC are strongly implicated in the generation of GnRH pulses. Data supporting this claim have come from multiple species included mice (Clarkson *et al.* 2017), goats (Wakabayashi *et al.* 2010; Wakabayashi *et al.* 2013) and sheep (Goodman *et al.* 2013; Li *et al.* 2015). At its core, the hypothesised GnRH pulse generator relies on the co-expression of NKB and Dyn (and their receptors) in ARC kisspeptin neurons, dubbed the KNDy hypothesis. (Goodman *et al.* 2007; Navarro *et al.* 2009; Lehman *et al.* 2010). The hypothesis stipulates that NKB acts in a reciprocal manner via its receptor on other KNDy neurons to drive pulsatility, Dyn acts as the ‘brake’ halting

pulses, and kisspeptin is the final output to GnRH neurons. Although we have not directly visualised or examined NKB or Dyn expression in kisspeptin neurons in our model, the high (almost 100%) co-expression of NKB and Dyn with ARC kisspeptin neurons (Goodman *et al.* 2007) does not seem to differ between adult female and male sheep (Cheng *et al.* 2010). Interestingly, the overall number of KNDy cells appears to be vastly greater in female sheep compared to males (Cheng *et al.* 2010). This sex difference is consistent with our relatively low numbers of kisspeptin neurons detected per section – similar to that of Cheng *et al.* (Cheng *et al.* 2010) – and may relate to the additional role of ARC kisspeptin neurons in the female sheep in facilitating the positive feedback induced GnRH surge (Estrada *et al.* 2006; Smith *et al.* 2009; Smith *et al.* 2011). The action of testosterone in rams is also a likely contributor to the low number of kisspeptin neurons detected, with negative feedback actions reducing kisspeptin output in intact rams versus castrated rams (Nestor *et al.* 2012).

Fig. 4. Representative photomicrographs demonstrating GnRH (red) and Fos (green) 645 labelling in rams 2 h post feeding. A-F: Examples of preoptic area GnRH neurons co- 646 expressing Fos in control (A-C) and lupin fed (D-F) rams (note: no co-expression was seen in 647 control). G-I: Fos immunoreactive cells in the mid ARC of control animals (G) and animals 648 fed lupins (H and I). 3V, third ventricle. Scale bars, 100 μ m.

Despite our data providing additional support for ARC kisspeptin, or KNDy, neurons facilitating the GnRH induced LH pulses – acting as the pulse generator, recent data have challenged this notion. In sheep, persistent LH pulses are seen in the presence of infused NKB receptor antagonists paired with a constant infusion of kisspeptin (Clarke *et al.* 2018), indicating KNDy neurons may not be the sole pulse generator in this species. In our study, we noted an overall increase in Fos activation in the mid ARC associated with increased pulse frequency. This is consistent with published data demonstrating that ARC kisspeptin neurons receive “upstream” input from ARC glutamatergic neurons (Ezzat *et al.* 2015) – although the majority of KNDy neurons themselves are glutamatergic (Cerneja *et al.* 2015; Merkley *et al.* 2015) – potentially contributing to the GnRH pulse generator.

Original studies investigating the supplemented feed effect in male sheep detailed an increase in GnRH pulse frequency within 6 hours of the acute increase in nutrition (Martin *et al.* 1994; Blache *et al.* 2003; Zhang *et al.* 2005; Blache *et al.* 2006), leading to an increase in testosterone and sperm production (Guan *et al.* 2017; Guan and Martin 2017). Zhang and colleagues (Zhang *et al.* 2004; Zhang *et al.* 2005) showed an increase within 6 hours in males (with a representative male showing a response at 3 hours). Our study showed a response potentially within 2 hours with mean LH significantly greater in supplemented animals at this time. Importantly, supplemented feed effects in male sheep are far more subtle and variable between animals – consistent with our relatively small changes in KNDy neurons – than other stimuli, such as the ram effect (De Bond *et al.* 2013; Fabre-Nys *et al.* 2017). This variability is evident in our kisspeptin-fos data, which only represents a single point in time and unfortunately cannot be directed paired to the overall LH activity during each period. Moreover, the neuronal link between hypothalamic-pituitary-gonadal (HPG) activity and nutritional cues is not fully understood. The LH response to supplemented feed is remarkably quick. Moreover, it is associated with no change in POA GnRH neuron number or activation, as determined by Fos immunohistochemistry, consistent with a previous report (Boukhliq *et al.* 1999). Remarkably, a subpopulation of GnRH neurons, limited to the medial-basal hypothalamus, show activation in rams following introduction to estrous ewes (Boukhliq *et al.* 1999) raising the possibility that a subset of GnRH neurons is responsible for GnRH pulses.

The rapid response also begs the question as to what the primary signalling factor from nutrition leads to the change in the hypothalamic KNDy neurons. It most likely involves insulin and leptin, two metabolic hormones that are known to affect reproduction (Blache *et al.* 2006). Importantly, plasma levels of insulin and leptin increase rapidly following supplemented feed in male sheep – within 3 h for insulin and there was a transient increase in leptin at 1 h (Zhang *et al.* 2004). Both of these hormones have known links to kisspeptin neurons. In mouse models, ARC kisspeptin neurons express the signalling leptin receptor (Smith *et al.* 2006) and the loss of leptin receptor, as well as insulin receptor signalling in kisspeptin neurons alters the onset of puberty (Qiu *et al.* 2013; Qiu *et al.* 2015). In sheep, leptin administration can restore *Kiss1* mRNA expression in the ARC following dietary restriction (Backholer *et al.* 2010). Alternatively, leptin and insulin could also regulate kisspeptin neurons indirectly in a neuroanatomical pathway involving pro-opiomelanocortin (POMC)/cocaine-amphetamine-regulated transcript (CART) and/or neuropeptide Y (NPY)/agouti-related peptide (AGRP) neurons (Backholer *et al.* 2010; Padilla *et al.* 2017). An indirect action of leptin on sheep KNDy neurons is also suggested by the absence of leptin-induced phospho-signal transducer and activator of transcription (pSTAT3) in these cells (Louis *et al.* 2011). We noted an overall increase in Fos activation in the mid ARC – possibly representing POMC/CART neurons, which are critical for regulation of energy homeostasis, but are also known to project to (Backholer *et al.* 2010; Cravo *et al.* 2011) and regulate kisspeptin neurons (True *et al.* 2013). Consistent with this, we also noted an increase in Fos expression in the dorsomedial hypothalamus (DMH), which is an area critical for leptin regulation of brown adipose tissue (BAT) temperature and the cardiovascular system (Fontes *et al.* 2001; Marsh *et al.* 2003; Horiuchi *et al.* 2006; Enriori *et al.* 2011; Rezai-Zadeh *et al.* 2014). Alternatively, other rapid nutrient signals, such a glucose or fatty acids, could signal to KNDy neurons either directly or indirectly and this warrants further investigation.

Overall, we have shown an acute increase in LH pulse frequency in response to nutritional supplementation in male sheep and are the first to implicate caudal ARC kisspeptin (KNDy) neurons (potentially acting as a GnRH pulse generator) in this effect. The results lead to the further understanding of the neuroanatomical pathways in the hypothalamus that mediate the response to feeding and link this to the reproductive system. This may be pertinent to anomalous energy balance links to infertility in humans. We conclude that KNDy neurons in the caudal ARC appear to mediate the increase in GnRH/LH production due to nutritional supplementation, further supporting the hypothesised role of the KNDy neurons as the GnRH pulse generator.

Conflicts of Interest

The authors declare no conflicts of interest.

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