

DR GREGROY GERALD CONDUCTIER (Orcid ID : 0000-0003-0834-9154)

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Corresponding Author Email ID: iain.clarke@monash.edu

Alteration in the relationship between tanycytes and gonadotropin releasing hormone (GnRH) neurosecretory terminals following long-term metabolic manipulation in the sheep.

Robert Pouchain Ribeiro Neto, Iain J. Clarke, Gregory Conductier

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Running title: Metabolic status alters GnRH terminals/tanycytes relationship

Abstract

The activity of the hypothalamo-pituitary gonadal (HPG) axis is influenced by energy reserves, such that an increase or a decrease in adiposity may perturb the secretion and action of GnRH. This is thought to be due to the signalling of hormones such as leptin, which act upon neuronal systems controlling GnRH secretion. Other work shows plasticity in the relationship between tanycytes and GnRH neurosecretory terminals in the median eminence across the estrous cycle and we hypothesised that similar plasticity may occur with altered metabolic status. We studied Lean, Normal and Fat ovariectomised ewes, which displayed differences in gonadotropin status and investigated the relationship between tanycytes and GnRH neuroterminals. In both Lean and Fat conditions, altered anatomical arrangement between these two elements was observed in the vicinity of the blood vessels of the primary plexus of the hypophysial portal blood system. These data suggest that such plasticity is an important determinant of the rate of secretion of GnRH in animals

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of differing metabolic status and this contributes to the relative hypogonadotropic condition that prevails with metabolic extremes.

Introduction

Reproduction is strongly influenced by metabolic status [1-3], especially a reduction of adipose tissue in anorectics, athletes and undernourished individuals, which disrupts menstrual cycles [4, 5]. This may be a conserved survival mechanism, to reduce energy expenditure associated with pregnancy and lactation. In sheep, food restriction of young animals delays puberty [6] and reduces gonadotropin releasing hormone (GnRH) secretion and gonadotropin secretion in ewes [2, 3, 6, 7]; [8]. On the other hand, in both rodents and humans, excessive energy stores due to either genetic [9, 10] or metabolic factors [11-13] also causes reproductive disorders and infertility. Whether the same or different mechanisms lead to perturbation of reproduction in lean and obese conditions is not known. Some key hormones link the metabolic state to reproduction, one being leptin [14, 15]. Thus, in lean ovariectomised (OVX) ewes, leptin treatment restored plasma LH levels [16, 17]. Leptin can also mitigate the effect of short-term starvation on plasma LH levels in women [14, 15] and leptin deficiency in ob/ob mice [9]. Other 'metabolic' hormones, such as ghrelin, may also play a role in the modulation of GnRH and gonadotropin secretion in various metabolic states [18-22] for review see [8].

In the sheep, as in other species, GnRH neuronal cell bodies are predominantly found in the preoptic area of the brain and project to the median eminence (ME) [23]. In the external zone of the ME, the GnRH neuronal fibers come into close association with the primary plexus of the hypophysial portal blood system and the neurohormone is secreted in a pulsatile manner [24, 25] to promote the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland [26-28, 29 Mercer, 1989 #323]. GnRH neurons, however, do not express leptin receptors, suggesting that nutritional information transmitted by this hormone is integrated through other cellular systems within the brain and then transmitted to the GnRH neurons [30, 31].

Many neuronal populations are involved in the control of GnRH secretion and may participate in the metabolic regulation the HPG axis (for review see [8]). In addition, glial cells are known to influence GnRH secretion within the ME. Among them, particular ependymogial cells, derived from the

embryonic radial glia and called tanycytes, are found within circumventricular organs (CVOs), including the ME. Some of these cells line the floor of the 3rd ventricle and send processes to the ME to be in close apposition to GnRH fibers [32-34]. During embryogenesis, tanycytes are thought to play a major role in the guidance of the GnRH axons into the ME and to establish functional neurosecretory units [35, 36]. Other studies in rodents indicate a crucial role for tanycytes as regulators of the dynamic release of GnRH throughout the estrous cycle [37, 38]. During the luteal phase, tanycytes end feet surround GnRH terminals forming a barrier between GnRH varicosities and the pericapillary space, limiting the secretion of GnRH into portal blood. With increasing estrogen levels and through an interaction with endothelial cells, these processes retract during the follicular/perioovulatory phase of the estrous cycle, allowing GnRH terminals to access the pericapillary space of the ME portal vessels [36-40]. This facilitates GnRH release at the time of the pre-ovulatory LH surge.

Another role for tanycytes is to regulate the blood-brain barrier [41, 42] and exchange between the cerebrospinal fluid compartment and blood at the level of the CVOs [43, 44]. In this regard, a role for tanycytes in the regulation of leptin entry to the brain has been described [45], which is dependent on the binding of leptin to its receptor and the activation of intracellular signaling pathways [45]. Tanycytes are also sensitive to ghrelin and are involved in its transport into the basal hypothalamus [46]. This strongly suggests that tanycytes may “sense” circulating levels of metabolic cues and transmit information to neurons.

Thus, extreme metabolic states (leanness or obesity) lead to either lowered or elevated leptin or ghrelin levels and this is associated with the disruption of the HPG axis [47]. Given that tanycytes display anatomical plasticity, are sensitive to leptin and ghrelin, and are crucial regulators of neurovascular junctions, we investigated the relationship between tanycytes and GnRH neuroterminals in the ME of Lean, Normal and Fat sheep. We hypothesized that plasticity of tanycyte end-feet is a determinant of the secretion rate of GnRH in these conditions.

Experimental Procedures

Animals: All animal procedures were approved by Monash University Animal Ethics Committee and performed according to the Australian Code for the Care and Use of Animals for Scientific Purposes. 15 adult ovariectomized Corriedale ewes were used. At the commencement of the protocol, the mean (\pm SEM) body weight of the ewes was 55 ± 1 kg. Animals ($n=5$ /group) were randomly assigned to Lean, Normal or Fat groups, generated by altered diet over 5 months. The Normal group was

maintained on a regular diet of pasture and lucerne hay *ad libitum*. The Fat group was fed lucerne hay *ad libitum* plus 1kg of lupin grain/week, whereas the Lean group was fed 400g lucerne hay/day. The animals were weighed weekly and target weights were reached by 4 months, after which the diets were maintained for another month. Blood samples (7ml) were taken every 10 minutes for 6 hours, via indwelling jugular cannulae, to measure plasma concentrations of LH, follicle stimulating hormone (FSH) and growth hormone (GH); the gonadotropin levels indicated the degree of hypogonadotropic condition in the Fat and Lean groups and the GH levels provided an indicator of metabolic status [3]. One week later, animals were euthanized with an overdose of phenobarbital (Lethabarb, 110 mg/kg; Virbac, Australia). The carotid arteries were cannulated and the heads perfused with 2L ice-cold (4 °C) saline (0.9% NaCl) followed by 1L fixative (4% PFA, 0.2% Picric acid in 0.1 M phosphate buffer [PB]) and finally with 1L fixative containing 20% sucrose. Hypothalami were dissected and post-fixed in fixative/sucrose for 24h. Cryoprotection was completed in 20% sucrose in 0.1M PB for 10 day and blocks were then frozen on dry ice and stored at -20 °C prior to sectioning. Blocks were randomly numbered so that the counting procedures were conducted blind.

Radio-immunoassays

Plasma LH concentrations were measured in duplicate, using the method of Lee et al. [48]. The assay sensitivity was 0.1 ng/ml, and the intra-assay coefficient of variation was <10% over the range of 0.6–15 ng/ml, and the inter-assay CV was less than 15.2%. LH pulse analysis (frequency and amplitude) was performed using a method described previously [49].

GH concentrations were measured by the method of Thomas *et al.* [3] using NIDDK-oGH-1–5 as standard and NIDDK-anti-oGH-3 antiserum. Assay sensitivity was 0.2 ng/ml. The intra-assay coefficient of variation (CV) was less than 10% between 0.98 and 89.4 ng/ml, and the inter-assay CV was less than 6.25%. GH values were used as indicators of the metabolic status of the animals, shown previously to be high in Lean animals and low in Fat animals [50]; mean values only are reported.

Immunostaining: Coronal sections (30µm) of the hypothalamus were cut at the level of the median eminence using a cryostat and the sections were stored in cryoprotectant (30% glycerol, 20% ethylene glycol in 0.1M phosphate buffer). Two sections from each animal were selected for immunostaining. All incubations were performed at room temperature unless otherwise specified. Sections were washed (3x10min) in 0.1M Tris-buffered saline (TBS), prior to an antigen retrieval step (sodium citrate 10mM, pH=9, 30min, 80°C). After cooling (20min), the sections were washed (TBS, 3x5min) and incubated in an antibody diluent/blocking solution (5% normal goat serum, 0.3% Triton X-100 in TBS; 1mg/ml sodium azide, 30 min). The sections were then incubated for 72h at 4°C with the primary antibodies diluted to the working concentration (Table1) in the blocking solution.

Primary antibodies used were raised against vimentin [51], a component of tanyocyte cytoskeleton; laminin, a marker for the pericapillary space; and GnRH (Table1). Since antibodies known to be specific for the staining of fenestrated capillary markers were not available for sheep, we stained the parenchymatous basal lamina of the capillaries with the laminin antibody [52]. Sections were then washed in (TBS, 3x10min) and secondary antibodies (Table 1) were applied in antibody diluent. The sections were finally washed (PB 0.05M, 3x10min), mounted on Superfrost Plus slides, coverslipped in fluorescence mounting medium (Dako, Agilent technologies Australia) and stored at 4 °C until acquisition of the fluorescent micrographs.

Microscopy: A Zeiss Axioscope Z1 fluorescence microscope equipped with a CCD AxioCam MRm camera and the ApoTome1 structured-illumination device was used to acquire images. Axiovision 4.8 software (Zeiss Inc., Lonsdale Australia) was used to manage the microscope and to acquire stacks of 20 multi-channel images (objective magnification 63X, z path = 0.260 μ m). Six pictures were randomly taken in the external zone of the ME for each animal.

Analysis, counting technique and statistics: Images were analyzed manually using the Axiovision software 4.8. Firstly, every immunopositive GnRH varicosity in each field was counted to provide the total number of GnRH varicosities (Figure 1). The images were analyzed using the “cut view” tool of Axiovision to avoid double-counting of the same varicosity and to allow for the discrimination of those which were potentially superposed. As the secretion of GnRH is influenced by plasticity of tanyocytes end-feet within the region of the capillary border [37], we then focussed on the number of vimentin processes and GnRH varicosities in the pericapillary area (less than 1 μ m from the laminin staining, Figure 2). Within the pericapillary area, we identified mainly two type of association between a GnRH varicosities, tanyocyte processes and/or laminin stained elements. On one hand, the varicosities were in direct contact with the laminin-stained tissue and these were designated “open varicosities”. On the other hand, vimentin processes were present between the laminin-labelled tissue and the varicosities and these were designated “closed varicosities” (figure 2). The presence of a GnRH varicosity within the pericapillary area and not in contact with a laminin stained element (parenchymal side of a blood vessel) was rarely seen without vimentin-stained processes between the two and these were not included in the analysis. The Axiovision cut-view tool was also used to further verify whether or not GnRH varicosities were surrounded by tanyocyte end-feet.

Statistics: All data are reported as means \pm SEM. One way ANOVA (body weight and abdominal fat), Two-way repeated measure (randomized blocks) ANOVA (numbers of varicosities, vimentin processes, “open” and “closed varicosities”) and two-way repeated measures ANOVA (gonadotropin

and GH profiles) followed by Holm-Sika's multiple comparison test compared the data sets between the 3 groups of sheep using GraphPad Prism. 7.01 for Windows.

Results

Body weight and hormonal status

The bodyweights of the Normal, Lean and Fat groups were 59.0 ± 0.84 kg, 35.2 ± 1.77 kg and 89.1 ± 1.77 kg, respectively ($p < 0.0001$). Abdominal fat weight was 2.0 ± 0.21 kg in Normal animals, 0.1 ± 0.02 kg in Lean animals and 5.6 ± 0.62 kg in Fat animals ($p < 0.0001$). Pulsatile LH secretion was altered by the dietary manipulation and the level of adiposity (Figure 3; Table 2). Two-way repeated measures ANOVA indicated that average plasma concentrations of LH differed between the groups ($p < 0.05$). Although the pulsatile pattern of LH secretion seemed differentially altered according to the nutritional state ("condition" x "time" interaction, $p < 0.05$), the average LH levels were significantly reduced only in the Lean group (Table 2). The amplitude of the pulses was not significantly affected by body condition and the pulse frequency was reduced in the Lean group only (Table 2). Notable, however, is some variability in the LH pulse patterns between animals within groups (Figure 3), with two Lean animal (778 and 759) having pulses of similar amplitude to the Normal animals and one Normal animal (679) having low LH values, compared to others in this group. LH pulse patterns in the Fat animals were quite variable. Average plasma FSH levels were higher in the Lean animals ($p < 0.05$) and not significantly altered in the Fat animals (Table 2). Average plasma GH concentrations were higher ($p < 0.05$) in Lean animals than in Normal animals and lower in the Fat group, although these differences did not reach statistical significance (Table 2).

Number of GnRH varicosities: Body condition had a significant ($p < 0.01$) overall effect on the number of GnRH immunopositive varicosities in the ME, being higher ($p < 0.01$) in the Fat animals (347.4 ± 52.7) than the Normal animals (220.6 ± 26.0) (Figure 4 and 5A). The number of GnRH varicosities in the Lean animals (191.8 ± 17.5) was similar to that of Normal animals ($p = 0.87$, Figure 4 and 5A).

Total GnRH varicosities in the pericapillary area: The total number of GnRH varicosities within $1 \mu\text{m}$ from the pericapillary space was altered by body condition, being increased ($p < 0.0001$) in the Fat sheep (Figure 4 and 5B). Considering that this could be due to the higher number of GnRH varicosities in the Fat animals (Fig 5A), we calculated the percentage of the total number of GnRH varicosities within the pericapillary space. This was similar in all groups (Fig 5C). We then determined the number of vimentin-immunopositive processes, 'open varicosities' and 'closed varicosities' within the pericapillary spaces.

Vimentin processes, open and closed varicosities: The staining obtained for vimentin was similar to that seen in rodents using the same antibody [41]. The number of vimentin processes in the pericapillary area (Fig 5D) was reduced ($p < 0.05$) in Lean animals and increased ($p < 0.001$) in Fat animals. The number of 'open varicosities' was significantly ($p < 0.0001$) affected by body condition (Figure 5E), being higher ($p < 0.0001$) in the Fat animals while there was no difference in the number of "closed varicosities" between groups (data not shown). However, the proportion of "open varicosities" was impacted by the nutritional status of the animals being increased ($p < 0.05$) in the Fat animals and reduced ($p < 0.05$) in the Lean animals.

Discussion

Matching reproductive activity to nutritional reserves (adipose) is fundamental to the survival of a species. Although the cellular and molecular mechanisms underlying the maintenance of energy homeostasis are extensively studied, the means by which metabolic status impacts upon the HPG axis is less well understood. It is generally known that many neuronal populations can "sense" the relative amount of nutrients or hormones that signal energetic status. More recent data point out to a crucial role of glial cells in such homeostatic regulation [53] (reviewed in [54]), including the ependymoglial cell tanocytes [45, 55, 56]. Previous studies have shown the importance of the plastic interaction between tanocytes, endothelial cells and GnRH varicosities in the regulation of the HPG axis function ([57, 58]). The present data suggest that such neuron/glia/vascular interactions is influenced by the metabolic status of animals affects the HPG axis.

Reduction in body weight has been shown previously to reduce plasma LH levels in OVX ewes [16, 59]. In the present study, this was also the case, although variability between animals was seen in all groups. Remarkably, the apparent reduction in plasma LH pulse amplitude in Fat animals (Figure 3) was not significant ($P = 0.11$), when compared to Normal animals, although variability across both groups was apparent. The statistical outcome would not be different with larger numbers of animals because of the assumption that variability would persist to the same degree. FSH levels were higher in the Lean animals, a finding which we have noted previously (unpublished data). This may be due to the lowered clearance of this hormone, which has a very long half-life of 1100 minutes in ovariectomized ewes [60], but we cannot rule out changes in the rate of synthesis or secretion. Certainly, reduced GnRH pulse frequency (reflected in LH pulse frequency in the Lean animals) favours increased FSH secretion [61]. As found previously [50], GH levels were increased in Lean and decreased in Fat animals (although not reaching statistical significance in our study) which is consistent with previous data showing altered GH clearance according to the level of adiposity in

monkeys [62] and sheep [63]. This also consistent with the known increase in ghrelin levels during negative energy balance state [64] and its stimulatory role on GH secretion [65].

Our data from Fat sheep indicate an accumulation of GnRH in the median eminence (increase in the density of varicosities) while the number of vimentin processes and the proportion of open varicosities (forming neurovascular junctions) is increased. In absence of significant alteration in the HPG axis of Fat sheep, this could be seen as a compensatory mechanism in order to maintain HPG axis function. However, this also suggests that reduced GnRH secretion leads to the accumulation of the peptide in the varicose fibers of the ME in animals that have been maintained in a Fat condition for months. This is in contrast to the farming process known as “flushing”, which refers to a *transient* increase in nutrition of ewes in order to improve reproductive performance, especially ovulation rate, conception rate and early embryo survival [66]. The mechanism of the “flushing” effect is not known, but may be due to effects at each level of the HPG axis; further studies to examine hypothalamic mechanisms to explain the “flushing” effect would be interesting. The long-term alteration of body weight that was employed in the present study is not pertinent to the “flushing” effect. Nevertheless, the increase in the number of “open” GnRH varicosities could reflect one of the way by which an increase in energy intake can improve reproductive function, but this would require further investigation. In the present study, the extended period of dietary manipulation may be regarded as creating a different metabolic state with different consequences in terms of brain function. Few data are available for the effect on the HPG axis of increased body fat beyond physiological levels for an extended period of time, although the effect on appetite regulation neuropeptides has been studied in sheep. Earlier data showed that the NPY gene expression is lowered in Fat sheep, while expression of genes for Kiss or POMC are unchanged [67] [68]. NPY is an inhibitor of GnRH secretion [69-71] while the melanocortins [72] and Kiss [73-76] are stimulatory. The influence of these factors on GnRH secretion may either increase or reduce the pool of GnRH in the ME, but our observations do not support this. Although no data are available in sheep, obesity is associated to a loss of responsiveness of the ARH neuronal systems to leptin [77, 78] and ghrelin [79, 80]. This would likely underpin the lack of activation of GnRH secretion in response to an increase in leptin levels from normal values to those that prevail in obesity [50].

In addition to the accumulation of GnRH in the ME of Fat sheep, there was also a clear increase in the number of open GnRH varicosities in these animals. This could compensate for the disturbance in the neuronal mechanisms responsible for GnRH release in order to maintain the peripheral LH levels. It is known from studies in rats, however, that one of the major regulators of GnRH secretion at the level of the median eminence is the free radical nitric oxide (NO) [39, 81, 82]. At the level of

the neurosecretory zone of the ME, the nitric oxide synthase enzyme (eNOS) can be found within the vascular endothelium [82]. NO facilitates GnRH secretion by inducing tanyocyte and endothelial plasticity leading to the formation of "open" GnRH varicosities. Leptin also induces the release of GnRH from ME explant in a NO dependent manner [83] and the use of fluorescent leptin in diet-induced obese mice suggests that the binding of the hormone to the endothelium in the ME is not altered [45]. In relation to our present data, it could be surmised that the functionality of the leptin/NO pathway to induce the remodeling of the ME is preserved in Fat sheep, but this requires further work. However, the higher number of vimentin processes in the pericapillary area of Fat animals suggests that the density of GnRH varicosities and tanyocyte end feet are correlated, but it does not influence the proportion of "open varicosities". This is consistent with our qualitative observation that the highest density of vimentin processes and GnRH varicosities are found in the same area of our sections independent of the presence of capillaries (data not shown). Whereas the GnRH neurosecretory terminals may be in a "ready to release" state, leptin/ghrelin resistance in neurons that control GnRH secretion may be the cause of impaired gonadotropic status. It is important here to note that ob/ob, db/db mice and fa/fa rats are obese because of the lack of either leptin or its receptor and these natural mutants are likely to present the same defects of the HPG axis than those of lean animals rather than those which are metabolically obese. Accordingly, ob/ob mice are hypersensitive to the metabolic effect leptin [84] and the HPG axis can be restored in these mice [9] as well as in humans carrying a mutation in the leptin gene [10] by leptin replacement, as it does in Lean sheep [16].

In the Lean sheep, GnRH content of the ME was similar to that in Normal animals. Nevertheless, the number of tanyocyte end feet and the proportion of "open varicosities" found in the pericapillary area was lower. In the Lean condition, levels of NPY are high and those of POMC (melanocortin) [85] and Kiss [86] are low, predisposing to reduced GnRH secretion [8]. The chronic energy deficient state is likely to cause a hypersensitivity to leptin in sheep [50] as observed in ob/ob mice [84]. Any significant increase in the circulating levels of factors able to lower NPY and increase melanocortins or Kiss activity/function (leptin, insulin, nutrients) may then trigger a very quick reversal of such inhibitory tone [16, 17, 86-89]. This would lead to an exaggerated release of GnRH. The NO/leptin hypothesis indicated above could also explain the reduction in the proportion of "open" varicosities. The lower leptin levels in Lean animals would reduce the production of NO from the capillaries, advancing tanyocyte process and preventing the contact between GnRH varicosities and the pericapillary space. This corroborates the hypogonadism observed in NOS-knockout mice [90]. Although such a mechanism has not been demonstrated in sheep, the reduced proportion of "open varicosities" in Lean animals indicate that this is a good model for such studies.

The effect of either leptin or ghrelin on the proportion of open varicosities and the molecular intermediates involved (i.e. activation of eNOS) remains to be determined. Collectively, our data suggest that the metabolic regulation of the HPG axis is likely to occur at least at two different levels. Firstly, the function of GnRH neurons may be altered and secondly, the arrangement of neurovascular junctions within the ME may determine the rate of GnRH secretion into hypophysial portal blood.

The authors of the manuscript have no conflicts of interest to declare.

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Figure legends

Figure 1: Fluorescent micrographs showing the counting technique to determine the total number of GnRH varicosity. The merged immunostaining of vimentin (tanycytes end feet, green), GnRH varicosities (white) and laminin (pericapillary space, red) is shown in **(A)**. The vimentin (green) then the laminin (red) staining were ‘hidden’ **(B and C respectively)** in order to reveal all the GnRH varicosities and improve the counting accuracy. The GnRH varicosities were counted manually (purple arrowhead), revealing 233 varicosities in this picture **(D)**. Each picture contains 20 images (z stacks), and each was analyzed in the three-dimensional perspective to avoid double-counting of the varicosities. Scale-bar = 5 μm .

Figure 2: Immunostaining detailing the discrimination between “open” and “closed” GnRH varicosities and the plasticity within the pericapillary space. (A) Outline of the pericapillary space; the GnRH varicosities, the “open” and the “closed varicosities”. The white dotted line contours the laminin staining (red) representing the boundaries of the pericapillary area within which the “open” and “closed” varicosities were counted. Purple arrows indicate GnRH varicosities (white) outside this boundary. The yellow arrows indicate typical “closed” varicosities showing tanycyte end feet (green) localized between the GnRH varicosity and pericapillary space (red). The purple arrowheads exemplify “open” varicosities, for which the GnRH varicosities are in direct contact with the pericapillary space. Scale bar = 5 μm

(B) An example of the discrimination between GnRH “open” and “closed” varicosities. In this example four consecutive slides (z levels, 0.260 μm apart) of one image was compounded. Initially (B₁), the GnRH varicosity (yellow arrow) was outside of the counting area (white dashed line) contouring the laminin staining (red). Gradually, the laminin staining moved towards the GnRH varicosity, evidencing pericapillary space plasticity (B₂-B₄), and becoming thick enough to contact the GnRH varicosity (B₃ and B₄). Then within the boundaries of the counting area (white dashed line) an “open” varicosity (purple arrowhead) was evident. Scale bar: 2 μm .

Figure 3: Alteration in bodyweight induces LH secretion disturbance. Patterns of LH secretion in Lean, Normal and Fat sheep over 6 hours. Arrow heads indicate defined LH pulses.

Figure 4: Vimentin, GnRH and Laminin immunostaining in the median eminence of sheep of altered body weight. Representative fluorescent micrographs showing immunostaining for vimentin (tanycytes process, green), GnRH varicosities (white) and laminin (pericapillary space, red) and the merged images for Normal, Lean and Fat animals. Note the visible increase in the total number of GnRH varicosities observed in Fat group. Scale bar = 5 μ m.

Figure 5: Quantification of the number of varicosities and the proportion of “open varicosities”. Quantitative analysis of the fluorescence micrographs, exemplified in Figure 4. Each point represents the average value obtained from 6 images for each individual animal. The horizontal bars represent the group averages and error bars are SEM. (A) The total number of GnRH varicosities counted in images independent of location with respect to the pericapillary space; this is increased ($p < 0.01$) in the Fat sheep. (B) A similar increase was seen when counting is performed within the pericapillary area, but the proportion of varicosities within the pericapillary area was similar between groups (C), suggesting that the increased number of GnRH varicosities within the ME of Fat sheep reflected an accumulation of the peptide in the ME varicosities. (D) The total number of vimentin immunopositive processes within the pericapillary area was reduced in Lean and increased in Fat animals. (E) The total number of “open” varicosities (neurohemal junctions) was greater in Fat animals than in Normal animals while (F) the proportion of “open” varicosities is reduced in Lean animals and increased in the Fat animals. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$ compared to Normal.

Peptides of interest	Primary antibodies Dilution	Origin	Secondary antibodies Fluorophore and Dilution	Origin
GnRH	Guinea-pig, polyclonal 1/2000	Gift from E. Hrabovski	Goat anti-guinea pig DyLight-550, 1/500	ThermoFisher #SA5-10095
Vimentin	Mouse, monoclonal (V9) 1/5000	Dako (M0725)	Goat anti-mouse DyLight-488, 1/500	ThermoFisher #35505
Laminin	Rabbit, polyclonal 1/1000	AbCam (Ab11575)	Goat anti-rabbit DyLight-647 1/500	ThermoFisher #SA5-10034

Table 1: Antibodies and dilutions used in the immunostaining procedure.

	Normal	Lean	Fat
LH parameters			
Average Plasma levels (ng/ml)	2.7±0.4	1.2±0.3*	1.8±0.2 ^{p=0.18}
Pulse amplitude (ng/ml)	2.3±0.5	1.3±0.4	1.3±0.3
Pulse frequency (pulse/h)	1.6±0.1	0.6±0.2***	1.4±0.1
Average FSH plasma levels (ng/ml)	4.6±0.9	8.4±0.9*	6.2±0.6
Average GH plasma levels (ng/ml)	1.3±0.5	5.8±1.66*	0.4±0.1 ^{p=0.5}

Table 2: Mean ± SEM

plasma LH, FSH and GH levels in Normal, Lean and Fat OVX ewes over 6h. *p< 0.05; ***p<0.001 compared to Normal. The P value for the Average LH and GH of Fat compared to Normal are also provided.

Figure 1

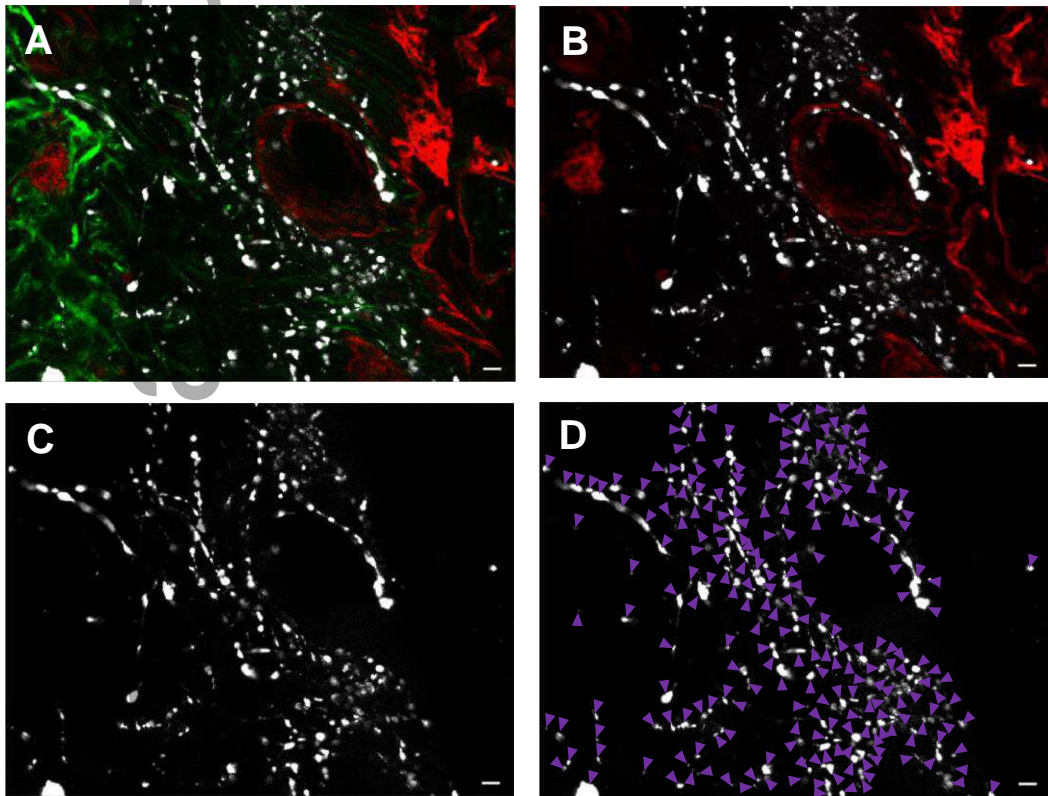


Figure 2

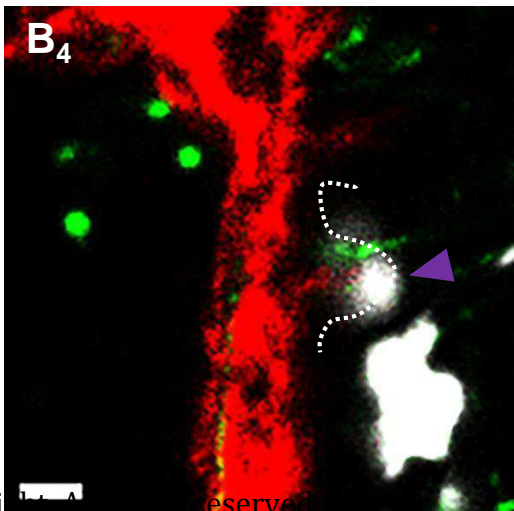
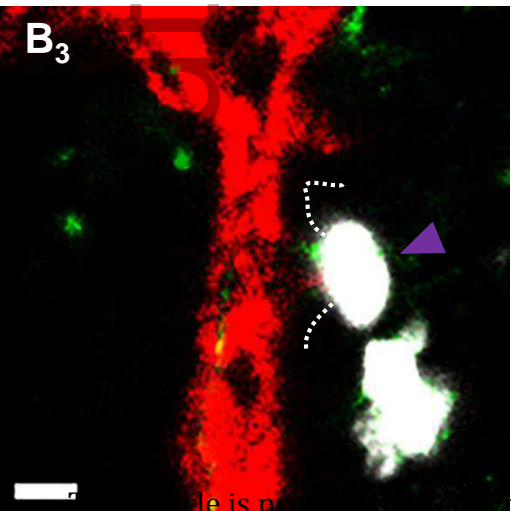
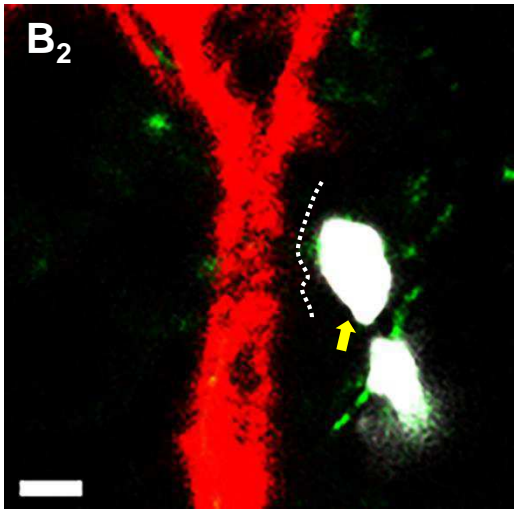
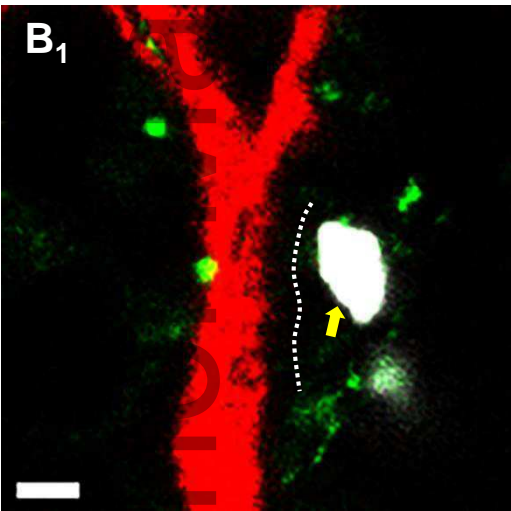
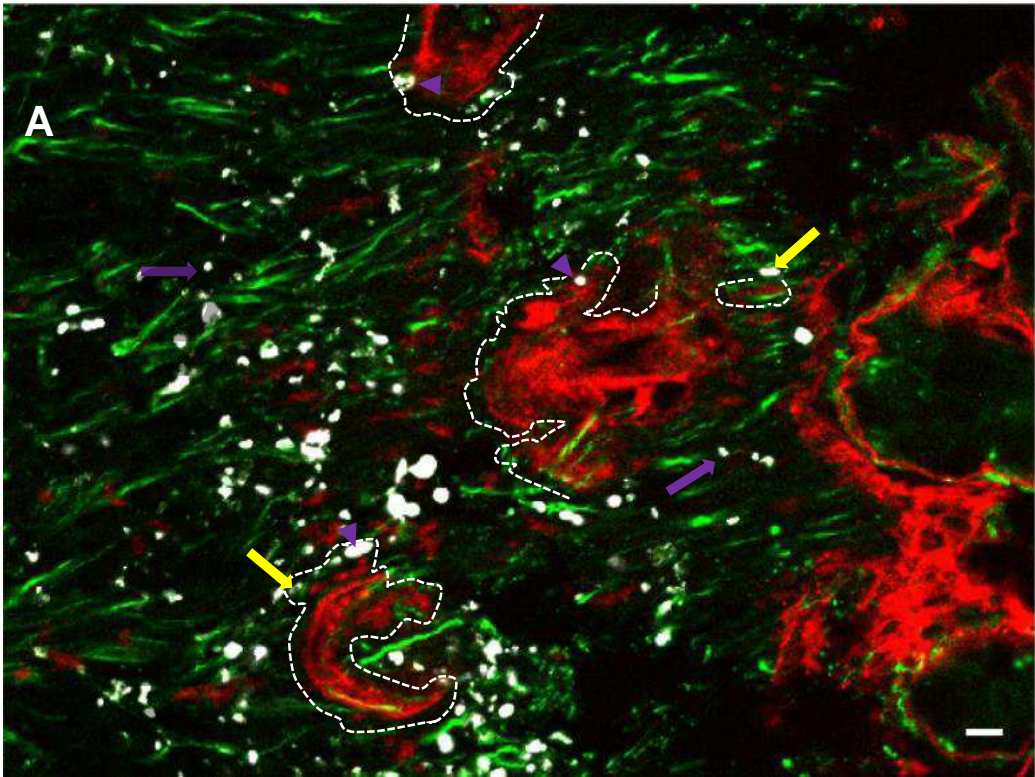


Figure 3

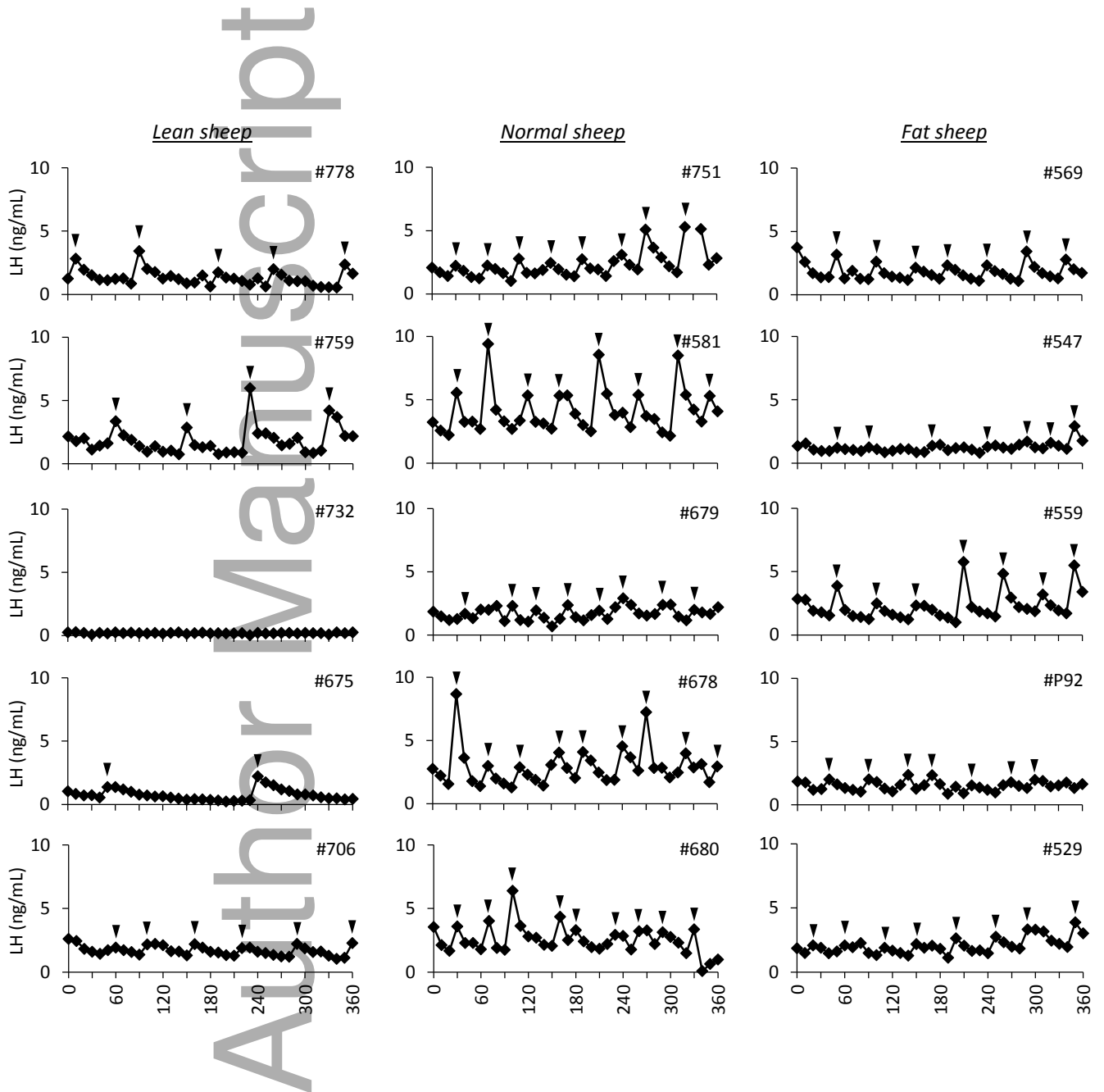
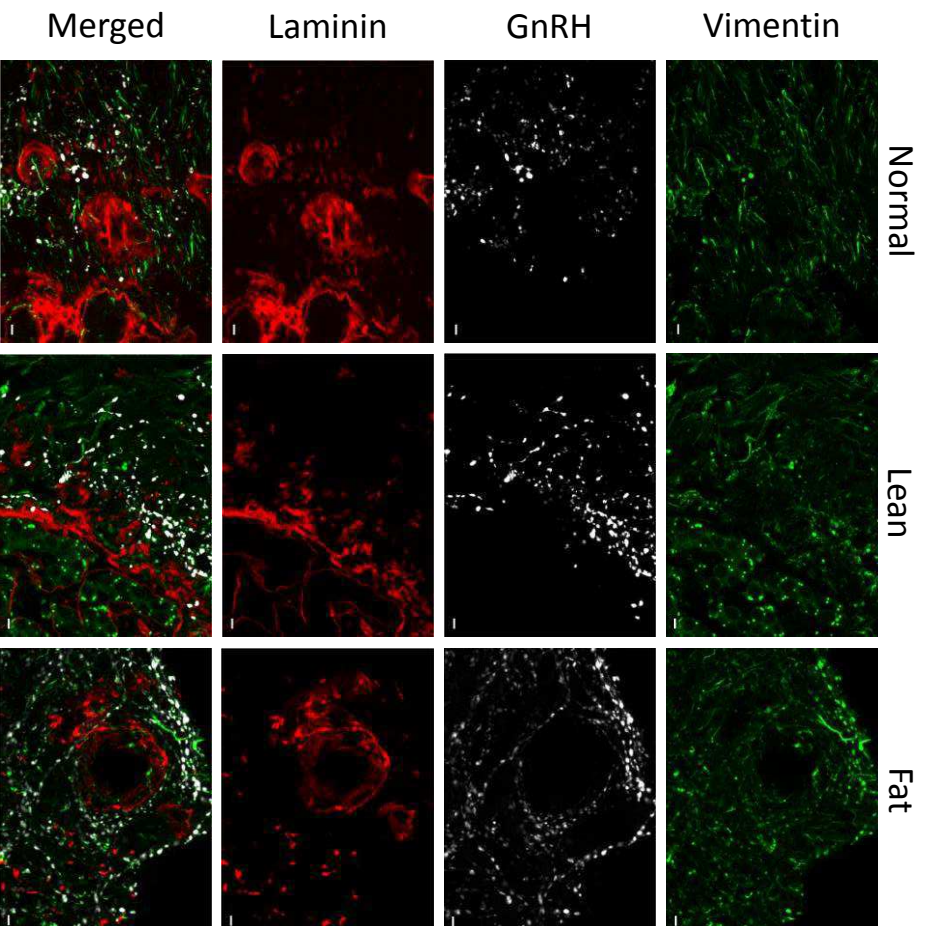
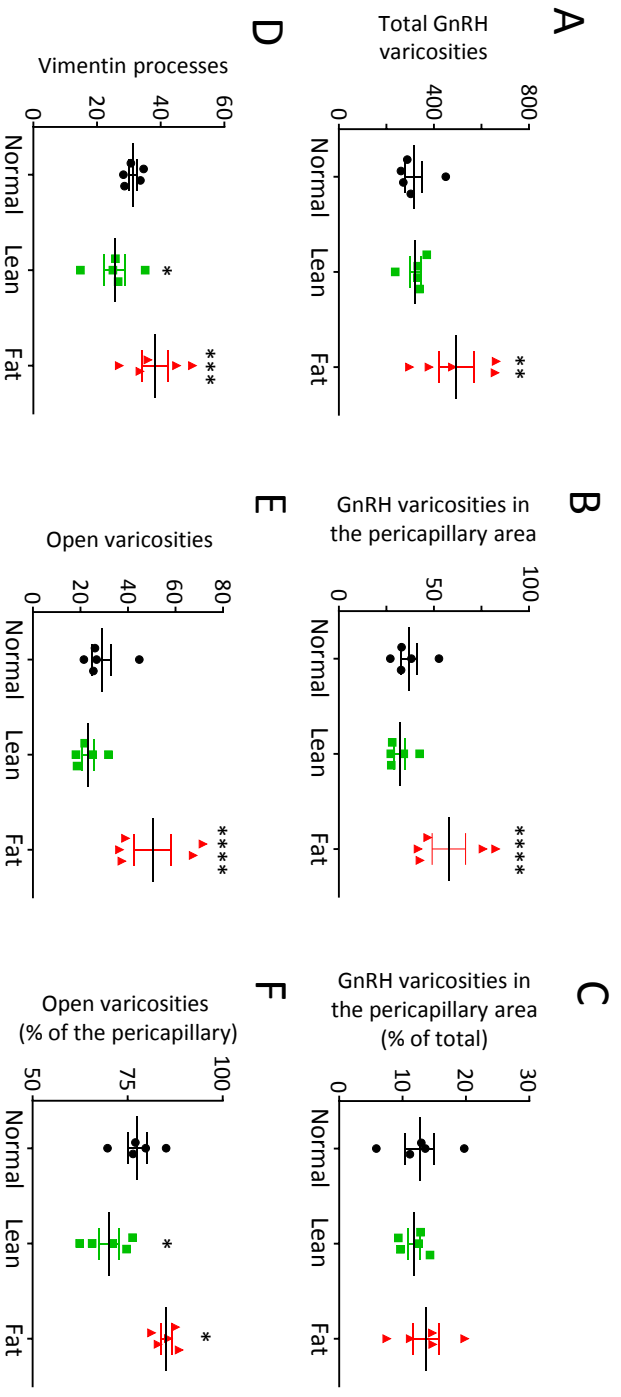


Figure 4



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Figure 5



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