

# Mechanisms regulating angiogenesis underlie seasonal control of pituitary function

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Edited by David W. Russell, University of Texas Southwestern Medical Center, Dallas, TX, and approved February 1, 2017 (received for review November 16, 2016)

Seasonal changes in mammalian physiology, such as those affecting reproduction, hibernation, and metabolism, are controlled by pituitary hormones released in response to annual environmental changes. In temperate zones, the primary environmental cue driving seasonal reproductive cycles is the change in day length (i.e., photoperiod), encoded by the pattern of melatonin secretion from the pineal gland. However, although reproduction relies on hypothalamic gonadotrophin-releasing hormone output, and most cells producing reproductive hormones are in the pars distalis (PD) of the pituitary, melatonin receptors are localized in the pars tuberalis (PT), a physically and functionally separate part of the gland. How melatonin in the PT controls the PD is not understood. Here we show that melatonin time-dependently acts on its receptors in the PT to alter splicing of vascular endothelial growth factor (VEGF). Outside the breeding season (BS), angiogenic VEGF-A stimulates vessel growth in the infundibulum, aiding vascular communication among the PT, PD, and brain. This also acts on VEGF receptor 2 (VEGFR2) expressed in PD prolactin-producing cells known to impair gonadotrophin secretion. In contrast, in the BS, melatonin releases antiangiogenic VEGF-A<sub>xxx</sub>b from the PT, inhibiting infundibular angiogenesis and diminishing lactotroph (LT) VEGFR2 expression, lifting reproductive axis repression in response to shorter day lengths. The time-dependent, melatonin-induced differential expression of VEGF-A isoforms culminates in alterations in gonadotroph function opposite to those of LTs, with up-regulation and down-regulation of gonadotrophin gene expression during the breeding and nonbreeding seasons, respectively. These results provide a mechanism by which melatonin can control pituitary function in a seasonal manner.

pituitary gland | angiogenesis | season | VEGF | melatonin

Pituitary hormone secretion regulates multiple functions in the body, including fertility, growth, fluid balance, and the response to stress. This regulation displays annual oscillations in most mammalian species, and is overtly seasonal in animals that have a tightly controlled reproductive window. It is thought that endogenous (i.e., circadian and circannual rhythm generators) and exogenous (i.e., photoperiod) cues can contribute to drive seasonal physiology (1). The duration of nocturnal release of the pineal hormone melatonin underlies the photoperiodic control of seasonality in sheep (2). As the synthesis and release of melatonin is inhibited by light, the longer nights of winter are associated with longer duration of melatonin production, whereas the opposite is true in the summer. Even though the reproductive cycle relies primarily on the secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus (3), one of the major sites of melatonin action is the pars tuberalis (PT) of the pituitary gland (4, 5). Indeed, melatonin is a regulator of endocrine function in the pituitary, and specifically inhibits prolactin-producing lactotrophs (LTs), known to be associated with repression of the reproductive cycle, directly within this tissue (6). However, the mechanism through which melatonin exerts this influence remains unclear.

Three functionally distinct regions of the pituitary gland, the PT, pars distalis (PD), and infundibulum, intercommunicate with one another via an elegant portal vascular arrangement. Although

melatonin is known to act on its receptors in the PT, LTs—the cells inhibited by melatonin—are exclusively found in the PD (7, 8). The mechanisms through which melatonin, acting in the PT, can control pituitary function in the PD are unresolved. Here we describe how the vascular arrangement in the infundibulum of the pituitary was drastically altered between the breeding season (BS) and nonbreeding season (NBS) in sheep. We therefore tested the hypothesis that melatonin could act to regulate pituitary function through the control of blood vessel growth and communication between the PT and PD.

Blood vessel growth (i.e., angiogenesis) is regulated by vascular endothelial growth factors (VEGFs), a family of peptides of which the most potently angiogenic and widely expressed is VEGF-A. Multiple VEGF-A products can be generated by alternative splicing of a single gene (9, 10). Alternative use of exons 6 and 7 will result in proteins of different length (e.g., 120, 164, or 188 aa in sheep, and 121, 165 or 189 aa in humans). Use of the proximal splice site in exon 8 generates the proangiogenic VEGF-A<sub>xxx</sub>a isoforms, and use of the distal splice site in exon 8 generates the VEGF-A<sub>xxx</sub>b isoforms, where xxx denotes the number of amino acids, and a or b denotes the carboxyl-terminal amino acid sequence. The most extensively studied and highly expressed isoforms of each family (VEGF-A<sub>165</sub>a and VEGF-A<sub>165</sub>b, respectively) are able to counteract the effects of each other on blood vessel growth (11). When VEGF-A<sub>165</sub>a binds VEGF receptor 2 (VEGFR2) on endothelial cells, it causes robust autophosphorylation and downstream signaling through phospholipase C, src, ras, and other pathways to induce a multitude of responses, including angiogenesis, vasodilation, increased vascular permeability, and cytoprotection

## Significance

Adaptation to seasonal changes in the environment is critical for survival in all species. In vertebrates, annual oscillations in pituitary hormones underlie the regulation of seasonal physiology. We found that, in sheep, the duration of pineal melatonin output at night controls the production of different forms of the protein vascular endothelial growth factor (VEGF) within a specific pituitary region, the pars tuberalis. Forms that block blood-vessel growth are made in winter, but those that stimulate it are made in summer. Further to the resulting remodelling of the vascular connection between the brain and pituitary, the temporally divergent VEGF-A variants operate as messenger signals on endocrine cells of a different part of the gland, the pars distalis, to regulate seasonal fertility.

Author contributions: D.O.B. and D.J.T. designed research; J.C.-M. performed research; J.C.-M., D.O.B., and D.J.T. analyzed data; and J.C.-M., D.O.B., and D.J.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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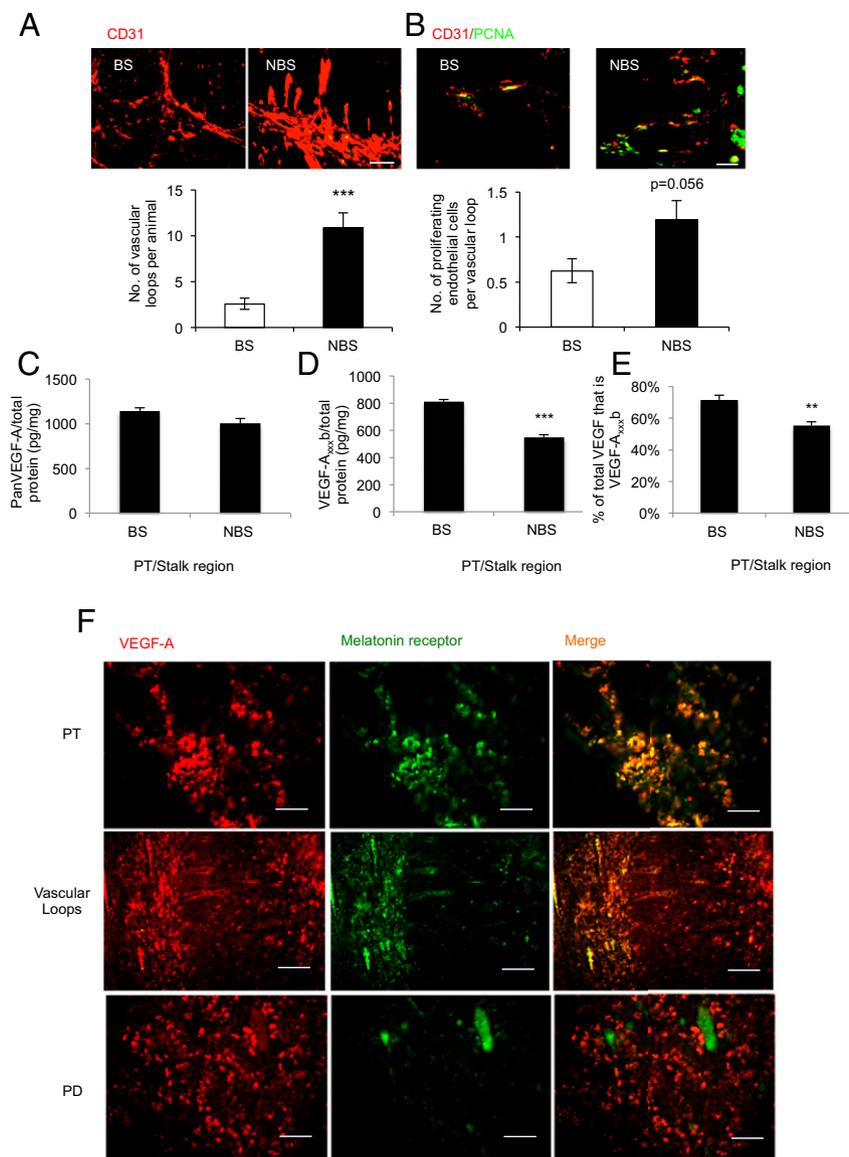
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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1618917114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1618917114/-DCSupplemental).

(12). In contrast, although the binding affinity for VEGFR2 is the same as that of VEGF-A<sub>165a</sub>, VEGF-A<sub>165b</sub> induces weak phosphorylation (13), does not bind the coreceptor Neuropilin-1 (14), which is responsible for intracellular trafficking and recycling to the membrane (15), and does not activate the full signaling pathway. This means it does not induce angiogenesis or vasodilation (11) or a sustained increase in vascular permeability (16), and results in VEGFR2 degradation, not recycling (15). It does, however, stimulate cytoprotection of endothelial and epithelial cells (17) and neurons (18). These two isoform families therefore have very different physiological consequences (19), but any differential role in seasonal pituitary angiogenesis is unknown.

We found that, whereas total VEGF-A was not altered between the BS and NBS, there was a dramatic switch in splicing in the BS from angiogenic VEGF-A<sub>xxx</sub>a isoforms to antiangiogenic VEGF-A<sub>xxx</sub>b isoforms in both the PT and PD. This was mirrored by a reduction in the number of blood vessels in the infundibulum.

Melatonin receptor expression in the PT and the infundibulum colocalized with cells expressing VEGF-A. Two potential mechanisms for VEGF-A-mediated regulation of pituitary seasonality are proposed here: (i) VEGF-A acting to regulate blood vessel function, which subsequently controls delivery of other hormones to the PD; and (ii) VEGF-A acting directly on LTs to control prolactin-associated down-regulation of the reproductive axis. We found that VEGFR2 colocalization with prolactin in the PD was increased outside the BS, consistent with a VEGF-A<sub>xxx</sub>a-mediated repression of fertility. In vitro culture of PT cells from BS sheep showed that duration of melatonin exposure controlled VEGF-A isoform secretion: long exposure induced VEGF-A<sub>xxx</sub>b production, whereas short exposure induced VEGF-A<sub>xxx</sub>a production. Culture of PT cells from the NBS revealed that melatonin given at frequencies seen in the winter could switch the expression of VEGF-A isoforms to BS levels. We then showed that PT cells treated with NBS melatonin regimens release VEGF-A<sub>xxx</sub>a, which



**Fig. 1.** Angiogenesis in the pituitary is seasonally dependent. (A) Endothelial staining in the PT/infundibulum and quantification of vessel loops in the summer (i.e., NBS) and winter (i.e., BS). (B) Endothelial proliferation (PCNA/CD31 double-positive cells) in the NBS and BS. (C) Total VEGF-A levels in the PT/stalk in the BS and NBS (not significantly different;  $P > 0.05$ ). (D) VEGF-A<sub>xxx</sub>b-specific ELISA on protein extracted from pituitaries of animals killed in the BS or NBS. (E) Proportion of VEGF-A that was VEGF-A<sub>xxx</sub>b in the BS and NBS. (F) Staining of the melatonin receptor (green) and VEGF-A (red) in different regions of the pituitary; costaining (found in PT and vascular loops) is shown as yellow ( $***P < 0.01$  and  $***P < 0.001$  vs. BS). (Scale bar: 50  $\mu$ m.)

directly induced prolactin secretion from PD cells. Finally, the time-dependent, melatonin-induced differential expression of VEGF-A isoforms resulted in alterations of gonadotroph function opposite to those of LTs in each season. Together, these results demonstrate that melatonin-mediated control of VEGF splicing could underlie intrapituitary regulation of seasonal fertility.

## Results

**Vascular Growth in the Pituitary Gland Is Seasonally Controlled.** To investigate the vascular architecture of the pituitary in a seasonally breeding mammal, we screened pituitary glands of sheep with the endothelial marker CD31. Staining showed a significant ( $P < 0.001$ ) increase in the number of vascular loops extending from the PT into the infundibulum in the summer (i.e., NBS) compared with animals culled in the winter (i.e., BS; Fig. 1A). To determine whether this was a result of endothelial proliferation in the NBS, we costained for CD31 and proliferating cell nuclear antigen (PCNA). We detected proliferating endothelial cells in both seasons, but a twofold increase in proliferating endothelial cells in the NBS (Fig. 1B). As angiogenesis is driven by VEGF, we measured VEGF-A in the pars tuberalis/stalk region of the pituitary. There was no difference in VEGF-A as measured by antibodies that detect all isoforms of VEGF-A (i.e., panVEGF; Fig. 1C). However, by using antibodies that specifically detect the exon 8b splice variants (VEGF-A<sub>xxx</sub>b), the expression of which has been shown to be antiangiogenic *in vivo*, a reduction in VEGF-A<sub>xxx</sub>b was measured in the NBS (Fig. 1D). This resulted in a change in the ratio of VEGF-A from 33% excess antiangiogenic isoforms in the BS to 60% excess angiogenic isoforms in the NBS (Fig. 1E). This indicates that the pituitary is in an antiangiogenic state in the BS, and suggests a link between day length and VEGF-A splicing.

To determine whether VEGF-A was expressed in the pituitary in cells that can respond to day length, we costained for melatonin receptor and VEGF. Fig. 1F shows that MT1 and VEGF-A were colocalized in the PT and, interestingly, also in the vascular loops (Fig. 1F, arrows) that connect the PT with the infundibulum. In contrast, whereas VEGF-A was expressed in the PD, MT1 receptors were not. The antiangiogenic isoforms had not previously been cloned from sheep, so we examined RNA expression by RT-PCR. Both isoforms were detected in pituitaries from sheep in both seasons (PD and PT; Fig. S1A). Cloning and sequencing of the PCR product confirmed that this was sheep VEGF-A<sub>xxx</sub>b (Fig. S1B).

The sheep sequence has a single nucleotide substitution compared with human DNA (a G in sheep, C in humans). This results in a single amino acid difference, with a sequence of SRTRKD instead of SLTRKD in human. Thus, the sheep VEGF-A<sub>xxx</sub>b isoforms are 1 aa shorter than the human ones. The cell type in which VEGF-A<sub>xxx</sub>b was expressed in the PT was identified by immunolocalization. Fig. S1C confirms that VEGF-A<sub>xxx</sub>b is expressed in the MT1-positive cells, which, in the PT, are not endothelial or glial-type folliculostellate (S100<sup>+</sup>) cells. These results suggested that melatonin could regulate expression of different VEGF-A isoforms in the PT, regulating angiogenesis in the pituitary in a seasonally dependent manner.

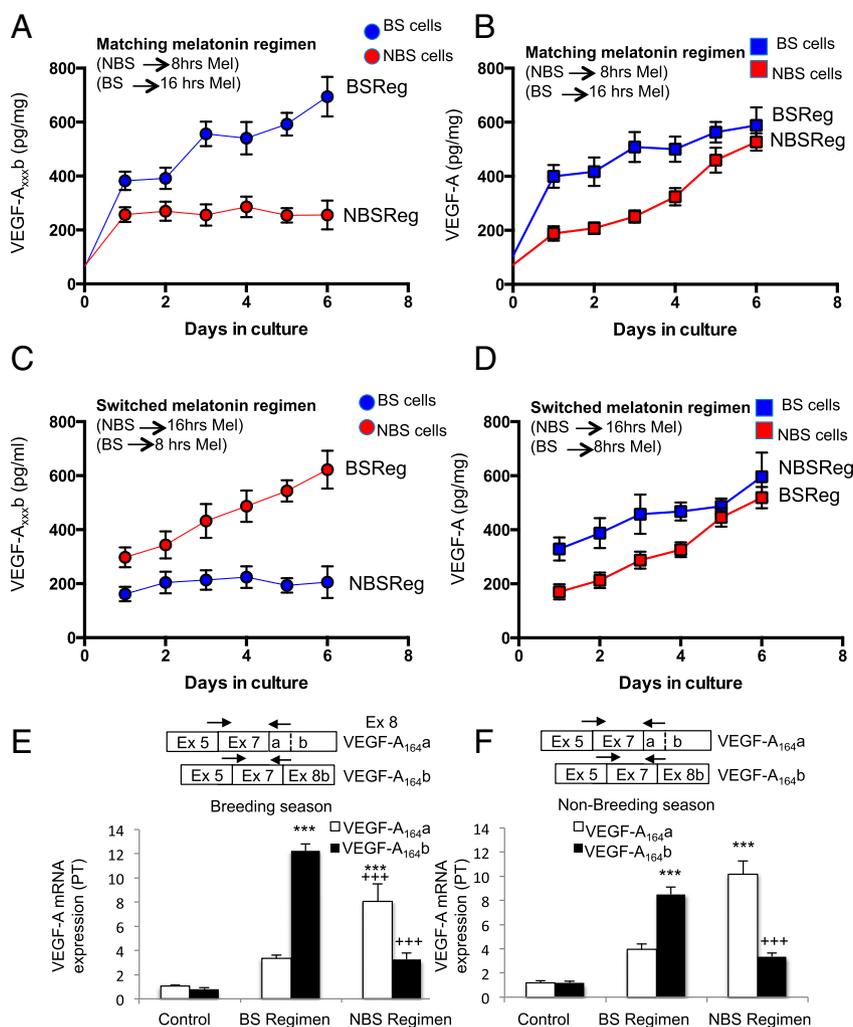
**VEGF-A Splicing Is Regulated by Duration of Melatonin Exposure in PT Cells.** We investigated VEGF-A isoform expression in cells isolated from the PT, which express the melatonin receptor and VEGF-A (Fig. S2A), by isoform family-specific ELISA and real-time PCR. Cells were cultured under conditions without melatonin (control; Fig. S2B) or with melatonin given for 16 h (mimicking winter, i.e., BS regimen), or 8 h (mimicking summer, i.e., NBS regimen). VEGF-A<sub>xxx</sub>b protein (Fig. 2A) was increased sevenfold by a BS melatonin regimen (Fig. 2A) in cells from BS animals (i.e., given the matching melatonin regimen), whereas panVEGF-A increased only fourfold (Fig. 2B). In contrast, when

cells from pituitaries of NBS sheep were given the NBS melatonin regimen, VEGF-A<sub>xxx</sub>b was increased only twofold (significantly lower than BS; Fig. 2A), but panVEGF-A was increased fivefold (Fig. 2B), although this response took longer than in the BS cells. This suggests that the length of time that the cells are exposed to melatonin on a daily basis controls the expression of the different splice variants of VEGF-A. It also suggests that NBS cells are less prepared to respond to melatonin exposure, as they take longer to increase their VEGF-A<sub>xxx</sub>a output.

To determine whether this was dependent on the stage of the annual reproductive cycle, we treated cells with a melatonin regimen that was reversed (i.e., opposite of that of the prevailing photoperiod) and found the same response for VEGF-A<sub>xxx</sub>b: BS regimen induced VEGF-A<sub>xxx</sub>b expression, whereas NBS regimen did not (Fig. 2C). The same was true for panVEGF-A (Fig. 2D): expression was induced by both regimens, but NBS cells were slower to respond than BS cells. To confirm that this was the result of a change in the RNA splice isoforms, we measured RNA levels of VEGF-A<sub>164</sub>b and VEGF-A<sub>164</sub>a by quantitative RT-PCR using isoform-specific primers. Fig. 2E shows that VEGF-A<sub>164</sub>a and VEGF-A<sub>164</sub>b were preferentially up-regulated by the NBS and BS regimens, respectively, in BS cells. In NBS cells, the same effect was induced by switching the melatonin regimen, indicating that this effect is specific to the duration of melatonin exposure, rather than the stage of the annual reproductive cycle from which the cell was sourced. These results indicate that melatonin can control angiogenesis protein production in the PT.

**VEGF-A Splice Isoforms and Receptors Are Present in the PD.** To determine whether VEGF-A could target endocrine and/or non-endocrine cells that are known to display seasonal plasticity, we screened the PD for VEGFR2. Costaining of VEGFR2 with folliculostellate cells (FSCs; Fig. 3A) and LTs (Fig. 3B) showed that VEGFR2 was colocalized with a proportion of FSC and LT, and, critically, that this colocalization increased ( $P < 0.01$  and  $P < 0.001$ , respectively) during the NBS, i.e., in the summer. There was also substantial VEGFR2 expression colocalized on endothelial cells in both seasons (Fig. 3C). Screening for proliferating endothelial cells indicated that there was more angiogenesis in the summer (i.e., NBS) in the PD (Fig. 3D), as well as the PT and infundibular stalk (Fig. 1D). Immunofluorescence staining for VEGF-A isoforms indicated that VEGF-A<sub>xxx</sub>b was significantly down-regulated and VEGF-A<sub>xxx</sub>a significantly up-regulated in the summer (i.e., NBS; Fig. 3E), providing a rationale for the up-regulation of angiogenesis in the PD. Quantification of the area of staining (Fig. 3F) confirmed this finding, as did quantitative ELISA for VEGF-A<sub>xxx</sub>b (down-regulation in the summer, i.e., NBS; Fig. 3G) and panVEGF-A (i.e., no change, and hence an implied up-regulation of angiogenic isoforms in the NBS; Fig. 3H).

**VEGF-A Isoforms Control Seasonal Endocrine Function.** These results led to two hypotheses: (i) that VEGF-A controls angiogenesis and this allows, by increased portal blood flow, an as yet unidentified compound to repress the reproductive axis (presumably, at least in part, by stimulating prolactin); and/or (ii) that VEGF-A itself is a signaling molecule from the PT to the PD, which directly contributes to the inhibitory regulation of the reproductive cycle by releasing prolactin. To test this latter hypothesis, we cultured PD cells from sheep in the NBS or BS and treated them with recombinant human VEGF-A<sub>165</sub>a (rhVEGF-A<sub>165</sub>a) or conditioned media from the PT cells taken at the same time of year, and measured prolactin production by RIA. Fig. 4A shows that VEGFR2 and prolactin were both expressed by PD cells in culture. Fig. 4B shows that the cells from both NBS and BS animals could be induced to release prolactin by thyrotrophin-releasing hormone (TRH), but not by melatonin. Fig. 4C shows that rhVEGF-A<sub>165</sub>a, given for the duration that matches NBS melatonin exposure (i.e., 8 h in the summer), resulted in significant prolactin release from PD cells



**Fig. 2.** VEGF-A isoforms levels are regulated by melatonin periodicity in the PT. (A) PT cells in culture were isolated from pituitaries of sheep and VEGF-A<sub>xxx</sub>b measured by ELISA. Cells from winter sheep (BS, blue) were treated with melatonin for 16 h each day for 6 d; cells from summer sheep (NBS, red) were treated for 8 h each day with melatonin. (B) Levels of panVEGF-A were also measured from these cells. (C) VEGF-A<sub>xxx</sub>b levels were measured from sheep PT cells incubated with the incongruous melatonin exposure for the time from which they were harvested (cells from summer sheep were given a winter melatonin regimen; those from winter sheep were given a summer melatonin regimen). (D) Levels of panVEGF-A from cells treated as in C. (E) VEGF-A isoform mRNA expression in PT cells from the BS (winter) after 6 d of treatment with BS and NBS melatonin regimens. (F) VEGF-A isoform mRNA expression in PT cells from the NBS (summer) after 6 d of treatment with BS and NBS melatonin regimens. Boxes show positions of the primers used to amplify the cDNA (\*\*\*)  $P < 0.001$  vs. control, +++  $P < 0.001$  vs. BS regimen).

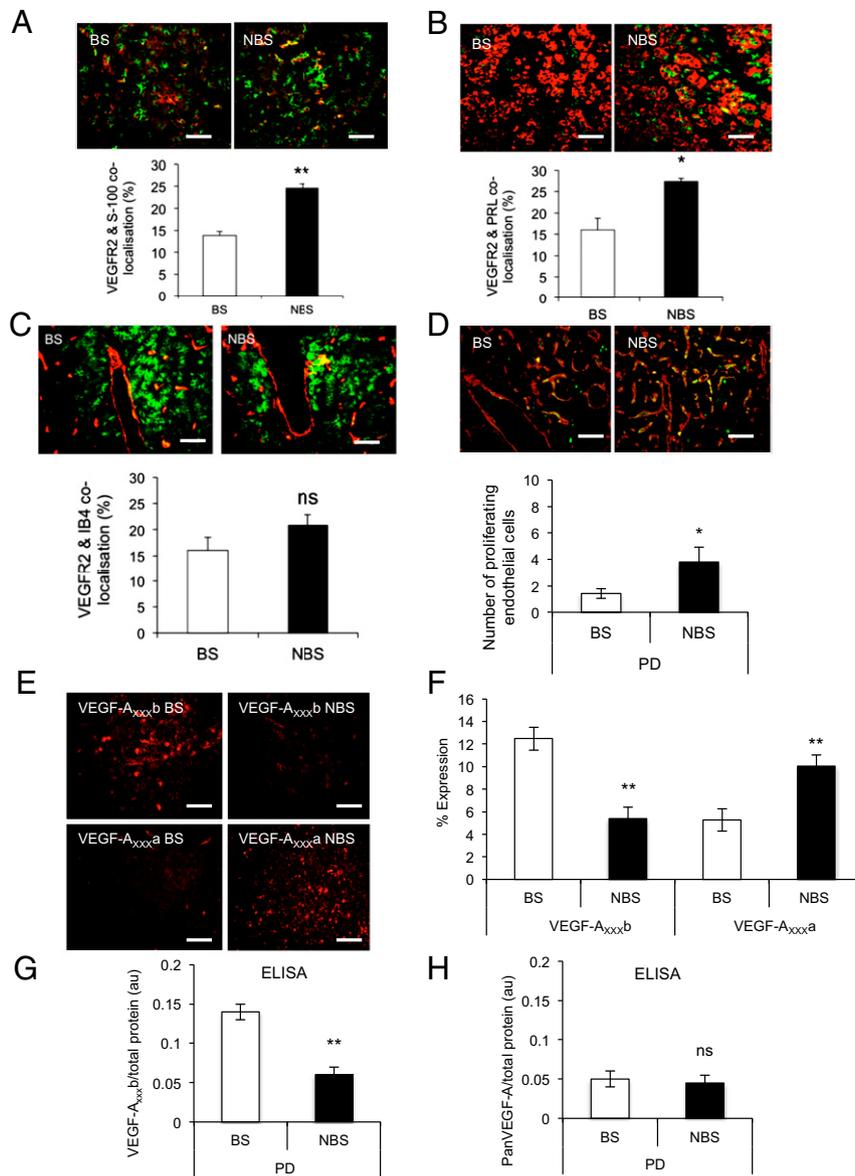
from NBS animals ( $P < 0.001$ ) and from cells from the BS (Fig. S3A). It also showed that rhVEGF-A<sub>165a</sub>, given at BS duration (16 h) did not cause prolactin release from BS (Fig. 4C) or NBS cells (Fig. S3A). We confirmed this at the RNA level (Fig. 4D and E).

To determine whether PT cells could generate VEGF-A isoform ratios that induced prolactin, we took conditioned media from the PT cells treated with melatonin and treated the PD cells with this conditioned media to mimic the in vivo situation. Conditioned media from PT cells treated with NBS melatonin regimen significantly stimulated prolactin protein (Fig. 4F) and mRNA (Fig. 4G) in cells from NBS ewes, but did not result in FSH production (Fig. S3B). Critically, this effect was completely blocked by an antibody to VEGF-A<sub>xxx</sub>a (Fig. 4F and G). Conditioned media from PT cells treated with BS melatonin regimen had no effect on prolactin production from BS PD cells (Fig. 4F), but did stimulate FSH mRNA production from these cells (Fig. S3C) and from cells from NBS ewes (Fig. S3D), an effect that was inhibited by pretreatment with a VEGF-A<sub>xxx</sub>b-specific antibody (Fig. S3D). This indicates that melatonin duration-induced

differential VEGF-A isoform production by the PT has the potential to regulate the seasonal production of prolactin and FSH by the PD through an intrapituitary paracrine mechanism mediated by VEGF.

## Discussion

The rationale for these studies was that dynamic and tightly regulated changes in the vascular communication between the brain and the pituitary gland could underlie seasonal physiology. To that end, we used a highly seasonal animal model, the sheep, with a well-characterized annual reproductive cycle. The results show that the pituitary microvasculature that connects the PT with the neural tissue of the infundibulum, before contacting the PD, displays dramatic seasonal remodeling, and that this could be in response to locally regulated splice variants of VEGF-A in the photoperiodic responsive/melatonin sensitive PT region. Importantly, we found that the melatonin-induced differential expression of VEGF-A isoforms in the PT throughout the annual reproductive cycle not only has the potential to alter the portal

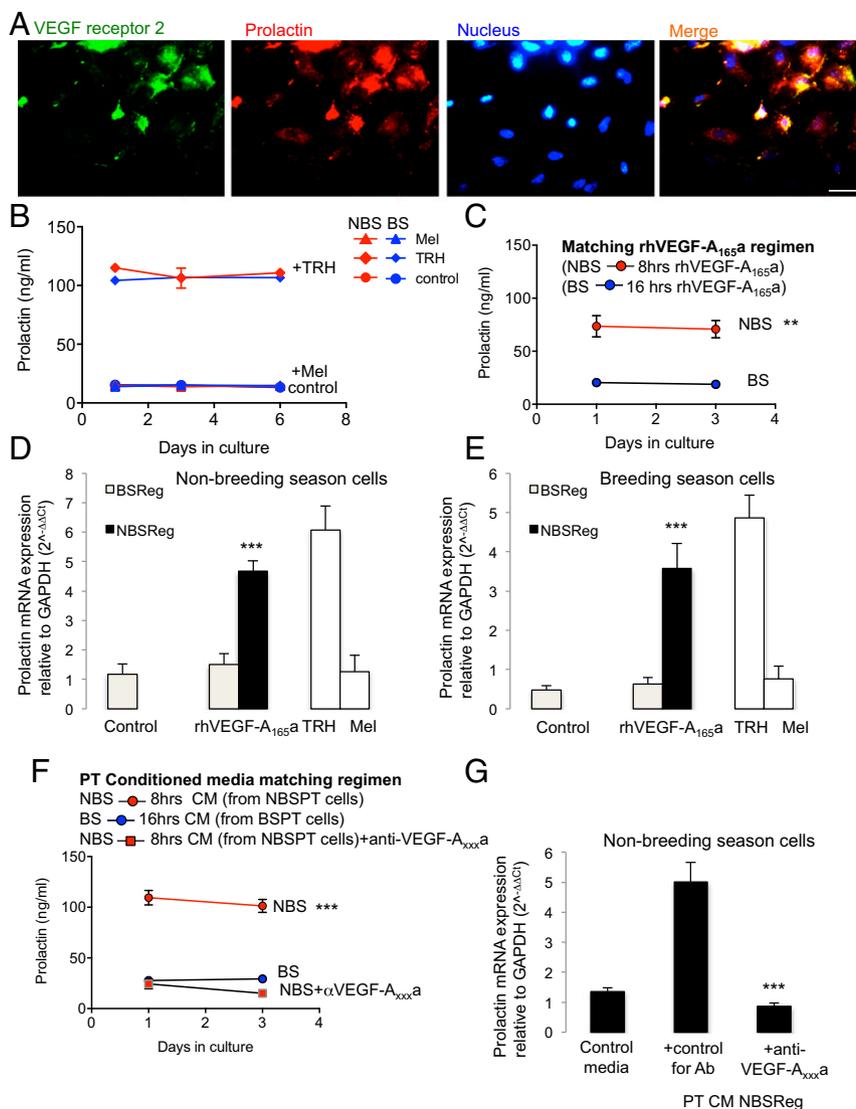


**Fig. 3.** VEGFR2 is up-regulated in the PD during the summer (i.e., NBS). (A) Colocalization of VEGFR2 (green) and glial-type FSCs (red) in the BS (winter) and NBS (summer). (B) VEGFR2 (green) expression in LTs (red) in the BS and NBS. (C) VEGFR2 (green) and endothelial cells stained by isolectin B4 (IB4, red) in both seasons. (D) Proliferating (PCNS, green) endothelial (IB4, red) cells were stained, and colocalization was quantified. (E) VEGF-A<sub>xxx</sub>b expression was detected in the PD in the winter (BS) but not in the summer (NBS); VEGF-A<sub>xxx</sub>a expression was detected in the summer (NBS) but not in the winter (BS). (F) Quantification of the expression of VEGF-A isoforms. (G) ELISA quantification of the amount of VEGF-A<sub>xxx</sub>b in the BS and NBS. (H) ELISA quantification of the amount of total VEGF-A in the two seasons (\* $P < 0.05$  and \*\* $P < 0.01$ ; ns, nonsignificant at  $P > 0.05$  vs. BS). (Scale bar: 50  $\mu$ m.)

microvasculature, but that it could itself operate as a messenger to modify the endocrine output from the PD. We established that the signal from the PT carried to hormone-producing cells in the PD to stimulate prolactin secretion and inhibit FSH during reproductive quiescence is the angiogenic isoform of VEGF, VEGF-A<sub>164</sub>. Moreover, the results reveal that the splicing of the VEGF-A pre-RNA from antiangiogenic VEGF-A<sub>164</sub>b in the winter (i.e., BS) to proangiogenic VEGF-A<sub>164</sub>a in the summer (i.e., NBS) results from the different duration of melatonin exposure, which occurs across seasons, highlighting the existence of a photoperiodically regulated system for the seasonal control of fertility.

The microvasculature of the pituitary gland is key to the regulation of multiple body functions because it controls the blood flow from the hypothalamus, altering the delivery of stimulatory and inhibitory signals to endocrine target cells (20). Here we

show that this vascular connection undergoes a remarkable seasonal adaptation throughout the annual reproductive cycle in response to an external cue, namely the changing photoperiod. Such vascular plasticity is manifested in alterations of endothelial cell proliferation that result in timely changes in the vascular loops that connect the PT with the infundibulum before giving rise to the long portal vessels that terminate in the PD. Even though these loops were first described more than six decades ago (21), the possibility that they could alter the connectivity between the brain and the pituitary at certain times of the year was not known. Although no apparent alterations in the ovine pituitary vasculature of male castrates exposed to different photoperiods was described in a recent report, detailed measurements were not undertaken and the vascular loops were not specifically examined (22). The increased number and surface area of these vascular loops during the long days of the NBS,



**Fig. 4.** VEGF-A mediates prolactin release from the PD in an isoform-dependent manner. (A) VEGFR2 expression in cultured prolactin positive cells (i.e., LTs) of the PD. (B) Prolactin secretion following treatment with TRH (positive control), melatonin (Mel, negative control), and medium (control) in PD cells cultured during the NBS (summer) and BS (winter). (C) Prolactin secretion in PD cells from sheep killed in the summer (NBS) after rhVEGF-A<sub>165a</sub> treatment was greater than that from winter (BS) sheep. (D) Prolactin mRNA expression in PD cells taken from ewes in the summer (NBS) following a summer (NBS) regimen of rhVEGF-A<sub>165a</sub> (8 h on, 16 h off) was greater than that of cells taken from the same animals following a winter (BS) rhVEGF-A<sub>165a</sub> regimen (16 h on, 8 h off). (E) In cells taken from ewes in the BS (winter), prolactin was induced only if VEGF-A was given in an NBS (summer) regimen. (F) Conditioned media from summer (NBS) PT cells treated with a summer (NBS) melatonin regimen (red) induced prolactin production. This was blocked by an antibody to VEGF-A<sub>xxx</sub>a. (G) Prolactin mRNA in summer (NBS) cells was induced in the presence of conditioned media from summer (NBS) regimen PT cells, and this was blocked by an anti-VEGF-A<sub>xxx</sub>a antibody (\*\**P* < 0.01 and \*\*\**P* < 0.001 vs. BS). (Scale bar: 20 μm.)

concomitant with an increase in endothelial cell proliferation, is in agreement with the increased number of cells proliferating in the PT shown in the present study and by another group (23) at this time of year. The afferent branches of these loops connect the photoperiodic-responsive PT with the FS cell-rich infundibulum, and efferent branches provide communication between the infundibulum and the PD, where most of the pituitary hormones are produced. Thus, the temporal remodeling of the vascular connection among these three tissues highlights the existence of a control point for seasonal physiology. The importance of this finding is supported by recent studies in rodents showing that the pituitary microvasculature adapts to the needs of pituitary endocrine cells and that it can control endocrine output according to the physiological requirement of the individual (24). This remodeling could substantially increase or decrease the transport of hormones from

the PT to the infundibulum and from the infundibulum to the PD, and is therefore able to accentuate or reduce the effect of hypothalamic-derived neuroendocrine signals, such as GnRH, to the hormone-producing cells of the pituitary. It is possible that this regulatory mechanism for the delivery of hypothalamic factors could operate in conjunction with the dynamic retraction/protraction of “endfeet” processes of tanycytes (25), the specialized ependymal cells of the glia, which have been shown to interact with the hypothalamic neuronal terminals and fenestrated capillaries of the median eminence and to play a role in the control of the seasonal reproductive cycle in birds (26). The VEGF isoforms differentially regulate fenestrations of endothelial cells (16), so it is possible that the two mechanisms could work together, although we are unable to use the evidence presented here to differentiate between dependent and independent mechanisms.

In other tissues, vascular remodeling and permeability is controlled by VEGF-A (19), so dynamic changes in the pituitary microvasculature were expected to correspond to alterations in VEGF-A expression. Indeed, VEGF-A-mediated changes in endothelial cell proliferation and angiogenesis were communicated in specific regions of the songbird brain across seasons (27). However, VEGF-A had been previously reported to remain unchanged in the pituitary of sheep under different photoperiods (28), and, in the present study, panVEGF-A expression did not differ between BS and NBS animals. Critically, the use of specific antibodies to the pro- and antiangiogenic isoforms of VEGF-A (these distinguish between isoform families, but not between the different length isoforms, hence VEGF-A<sub>xxx</sub>a and VEGF-A<sub>xxx</sub>b) revealed differential isoform expression between the long days of the NBS and the short days of the BS, with overexpression of antiangiogenic VEGF-A<sub>xxx</sub>b variants in the BS and increased expression of proangiogenic VEGF-A<sub>xxx</sub>a variants in the NBS, providing an explanation for the observed changes in the microvasculature. As the PT is the tissue with the highest density of melatonin receptors (4, 5), and reliably translates the effects of photoperiod on circadian and circannual physiology within the pituitary (1, 29), our results showing the coexpression of VEGF-A and melatonin receptors in PT-specific cells provide compelling evidence that the seasonal regulation of the vascular connection between the brain and the pituitary gland is mediated by a melatonin-induced mechanism within the PT region that leads to differential expression of pro- and antiangiogenic VEGF-A isoforms.

Moreover, our results show that, in addition to the PT-specific cells, melatonin could also act directly on the vascular loops, revealing a target for melatonin action to translate photoperiodic effects on seasonal physiology. As the blood flows from the brain to the pituitary (11), alterations in the vascular loops of the infundibulum that will give rise to the long portal vessels (12) could contribute not only to regulate the transfer of PT products to the PD, but also to alter the delivery of hypothalamic factors; thus, the increased vascular connections during the long days of summer would be expected to favor increased supply of stimulatory and inhibitory hypothalamic signals to the PD at this time of year. Notwithstanding that, the reduction in vascularity during the short days of winter is likely to play a role in the modulation of the gonadotroph response to GnRH by means of preventing desensitization of GnRH receptors (30) and fine-tuning the differential control of gonadotropin secretion (31, 32), which are essential processes to ensure normal fertility.

Photoperiodic information is encoded by the duration of nocturnal melatonin secretion (2), so we used a paradigm whereby ovine PT cells were cultured and exposed daily to summer (i.e., NBS) or winter (i.e., BS) durations of melatonin treatments (8 h vs. 16 h, respectively) over a period of 6 d. PT cells from the same animals were exposed to the matching and nonmatching (i.e., opposite season) melatonin regimens, so we were able to differentiate direct effects of the melatonin signal and those resulting from its interaction with the circannual phase. We show that duration of melatonin exposure induced a striking differential expression of VEGF-A isoforms, with up-regulation of the proangiogenic isoform VEGF-A<sub>xxx</sub>a by a short-duration regimen (i.e., 8 h, summer; NBS) and up-regulation of the antiangiogenic isoform VEGF-A<sub>xxx</sub>b by a long-duration regimen (i.e., 16 h, winter; BS). This melatonin duration-dependent differential expression of VEGF-A isoforms was also recorded in cells obtained in the opposite season but at a slower rate, highlighting the requirement of PT cells to be entrained to the new signal. Thus, the results are consistent with the findings *ex vivo* and demonstrate that pituitary microvascular remodeling is likely to be sensitive to the changing photoperiod and adapts to the physiological requirements of the animal in response to time-dependent melatonin signals acting on VEGF-A. The mechanism through which melatonin switches splicing of the VEGF-A gene is not yet known, but alternative splicing of VEGF has been shown to

be regulated by activation of the RNA binding proteins SRSF1, SRSF2, and SRSF6 by the kinases SRPK1 and Clk4 (33).

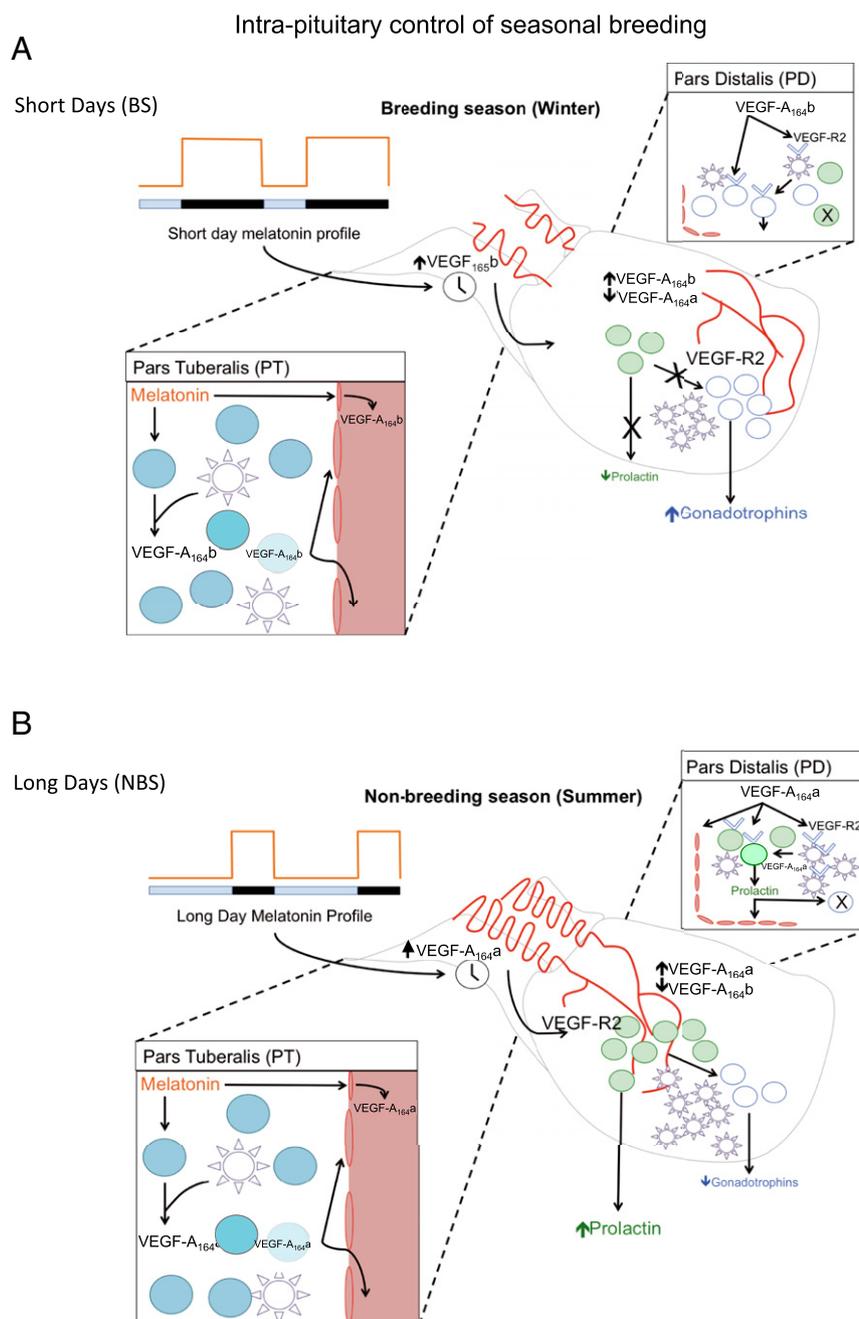
We then investigated whether the seasonal regulation of VEGF-A in the PT could affect the function of the PD. We show that VEGF-A receptors are expressed in endocrine, endothelial, and FS cells in the PD, and that their colocalization is also under seasonal control, with up-regulation during the long days of the NBS. In addition, there was increased content of proangiogenic VEGF-A isoforms at this time of year and, conversely, increased content of the antiangiogenic isoforms during the short days of the BS. As the seasonal regulation of VEGF-A isoform expression was shown to be melatonin-dependent and, in accordance with previous studies (34), the PD was shown not to contain melatonin receptors, the varying content of VEGF-A isoforms in the PD is likely to rely on a paracrine mechanism (35). The physiological significance of this was first revealed in the PD microvasculature, with increased endothelial cell proliferation demonstrated during the NBS. We show that this increase in angiogenesis at this time of the annual reproductive cycle is concomitant with an increase in the prevalence of FS cells containing VEGF receptors. FS cells are glial-like, nonendocrine cells that, via gap junctions, generate a 3D network throughout the pituitary to coordinate its function (36, 37). These cells secrete an array of paracrine factors known to influence endocrine cells such as gonadotrophs and LTs, and are a primary source of VEGF-A (35). In seasonal breeders, FS cells are distributed throughout the PD and PT (38) and respond to photoperiodic changes with a high degree of plasticity (39, 40). In sheep, significant ultrastructural changes, together with enhanced number of intercellular adherens junctions and increased number of elongated processes surrounding endocrine cell clusters, were reported during the long days of the NBS (41). As FS cells do not contain melatonin receptors (42), our findings revealing up-regulation of VEGF receptor content in these cells at this time of year provide evidence for a role of VEGF-A in the dynamic changes of the FS cell network to control vascular plasticity via the regulation of its own production during the annual reproductive cycle. The seasonally regulated differential expression of VEGF-A isoforms in the pituitary gland of a short-day breeder unraveled here could also operate in long-day breeders, such as hamsters and horses, as part of the mechanisms controlling their annual physiology. Indeed, preliminary results have provided evidence that, in thoroughbred horses, VEGF-A isoform expression in the PT and PD regions of the pituitary is also seasonally regulated (43), suggesting that this is a conserved mechanism for seasonal adaptation in photoperiodic mammals.

Notably, we show that, in addition to its actions on the pituitary vasculature and FS cell population, VEGF-A has a potent prolactin releasing effect, and that this stimulation depends on time of exposure of the ligand and density of VEGF receptors in LTs, which is increased during the long days of the NBS. Moreover, these stimulatory effects of VEGF-A on prolactin synthesis and release were accompanied by suppression of the gonadotrophic axis, as revealed by inhibition of FSH gene expression. Melatonin was shown to mediate the photoperiodic regulation of prolactin secretion through a direct action within the pituitary gland (6). Because MT1 melatonin receptors are selectively expressed in the PT, and this region is deprived of LT cells (7, 8), a paracrine mechanism for the control of prolactin secretion from the PD is warranted. Activation of MT1 melatonin receptors in the PT is known to inhibit adenylyl cyclase, and pharmacological studies in sheep have shown that melatonin impairs forskolin-induced hypersecretion of cAMP, with inhibition of prolactin from the PD through the reduction of a paracrine signal (44). However, although several compounds such as tachykinins, substance P, and neurokinin A are produced by the PT and can stimulate prolactin release (45–47), characterization of the chemical identity of that signal has been elusive. Here we show that the stimulatory effects of VEGF-A on prolactin were mimicked by conditioned media from PT cultures exposed to an NBS regimen

of melatonin, and that these actions of PT media were blocked by a specific VEGF-A<sub>xxx</sub>a antibody, demonstrating that VEGF-A is a potential paracrine signal, and that melatonin-induced differential VEGF-A isoform production by the PT can regulate the seasonal production of prolactin and FSH.

Because these effects were also recorded in PD cells obtained in the opposite season (i.e., BS), albeit with a 3-d lag required for adaptation, our results show that the photoperiodically induced

paracrine mechanism mediated by VEGF-A can ultimately override the circannual phase of the PD target cells, and entrain it to the new photoperiod. The increased VEGF receptor content in the PD during the NBS plays a major role in mediating this process, and thus in the biological adaptation to a summer physiology, because VEGF-A treatments mimicking an NBS melatonin regimen showed a delayed response in BS cultures in which the VEGF receptor content was reduced. Entrainment of the PD cells to a



**Fig. 5.** Working model for a melatonin-induced, VEGF-A isoform-dependent intrapituitary regulation of seasonal physiology. In this model, the duration of nocturnal melatonin secretion induces differential synthesis and release of proangiogenic and antiangiogenic isoforms of VEGF-A in the PT region of the ovine pituitary and in the vascular loops that connect this tissue with the infundibulum. (A) In the short days of winter (BS), the long duration of nocturnal melatonin exposure up-regulates the secretion of the antiangiogenic isoform VEGF-A<sub>164b</sub> at the expense of the proangiogenic isoform VEGF-A<sub>164a</sub>, resulting in reduced angiogenesis, reduced density of VEGF receptors in endocrine and FS cells of the PD, suppression of prolactin secretion, and no inhibition of the gonadotrophic axis. (B) In contrast, during the long days of summer (NBS), the short duration of nocturnal melatonin exposure up-regulates the secretion of the proangiogenic isoform VEGF-A<sub>164a</sub> at the expense of the antiangiogenic isoform VEGF-A<sub>164b</sub>, leading to increased angiogenesis, increased density of VEGF receptors in endocrine and FS cells of the PD, stimulation of prolactin secretion, and inhibition of the gonadotrophic axis.

specific phase of the circannual cycle explains why NBS cells failed to secrete prolactin in response to the first 8 h of a BS (16 h) VEGF-A regimen or PT-conditioned media from the BS.

In rodents, melatonin-induced suppression of cAMP is followed by sensitization of adenosine A<sub>2b</sub> receptor signaling, leading to subsequent increase in cAMP and cAMP response element binding protein (CREB) phosphorylation (28). Disruption of this signaling pathway in MT1 melatonin receptor-KO mice resulted in altered prolactin secretion, implicating cAMP and adenosine in this biological response to melatonin. Our results indicate that VEGF-A is likely to be downstream of that pathway to bring about the biological response. Indeed, cAMP signaling, CREB phosphorylation, and adenosine are associated with angiogenesis (48, 49) via stimulation of VEGF-A (50), and, whereas pharmacologically induced cAMP up-regulation and treatment with adenosine stimulated VEGF-A expression in smooth muscle cells (50), the selective knockdown of all VEGF-A isoforms blocked the actions of elevated cAMP on hippocampal neurons (51). The melatonin-induced VEGF-A regulation of prolactin secretion shown in this study will have an impact on the gonadotrophic axis in addition to its direct inhibition of FSH, because, when combined with dopamine, prolactin impairs the gonadotroph response to GnRH in a seasonally dependent manner in long- and short-day breeders (52–54).

Our results provide evidence for an intrapituitary mechanism that responds to an external independent signal to regulate seasonal physiology. We propose a model whereby the duration of nocturnal melatonin secretion promotes alternative splicing of the VEGF-A gene, leading to differential synthesis and release of proangiogenic and antiangiogenic isoforms of VEGF-A within the PT region of the pituitary gland and in the vascular loops that connect this tissue with the infundibulum (Fig. 5). The resulting output of VEGF-A isoforms will have two complementary effects: (i) it alters the temporal vascular connection between the brain and the pituitary gland and (ii) it can be used as a paracrine signal to modify the seasonal activity of endocrine cells in the PD that control reproduction. In this model, the long duration of nocturnal melatonin exposure during the winter up-regulates the secretion of antiangiogenic isoforms VEGF-A<sub>xxx</sub>b at the expense of proangiogenic isoforms VEGF-A<sub>xxx</sub>a, resulting in reduced angiogenesis, reduced density of VEGF receptors in endocrine and FS cells, suppression of prolactin secretion, and no inhibition of the gonadotrophic axis characteristic of the BS. Conversely, the short duration of nocturnal melatonin exposure during the summer will up-regulate the secretion of proangiogenic isoforms VEGF-A<sub>xxx</sub>a at the expense of antiangiogenic isoforms VEGF-A<sub>xxx</sub>b, leading to increased angiogenesis, increased density of VEGF receptors in endocrine and FS cells, stimulation of prolactin secretion, and inhibition of the gonadotrophic axis, characteristic of the NBS. Thus, the model permits a physiological adaptation to the seasonal requirements of the species by means of an angiogenesis-dependent intercommunication between two regions of the pituitary.

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## Materials and Methods

Details of standard protocols are given in *SI Materials and Methods*. Ovine pituitary glands were obtained from ovary-intact females during the BS (i.e., December/January) and the NBS (i.e., June/July). Animals were killed for commercial reasons at an abattoir, and pituitaries were removed immediately after death. During the BS, ewes were confirmed to be sexually active on the basis of a recently formed corpus luteum (CL) together with the presence of a large follicle (>2 cm). By contrast, in the NBS, ewes were considered to be anestrus when no CL but a corpus albicans was observed in the gonad, and follicles present were <2 mm in diameter.

**Expression Studies.** Pituitaries were stained and RNA was extracted (55) by using standard procedures (antibodies are detailed in *Tables S1* and *S2* and primers are shown in *Table S3*). The term “VEGF-A<sub>xxx</sub>b” is used because the antibodies do not distinguish between the different VEGF-A<sub>xxx</sub>b isoforms (e.g., VEGF-A<sub>121</sub>b, VEGF-A<sub>165</sub>b, VEGF-A<sub>189</sub>b). The term “VEGF-A<sub>164</sub>b” or “VEGF-A<sub>165</sub>b” is used when the methodology specifically describes the sheep 164-aa isoform (isoform-specific RT-PCR, as the forward primers cross exon 5 and exon 7, or the human 165-aa isoform when recombinant protein is used).

**Primary Cell Cultures.** Ovine primary pituitary cultures were produced by careful dissection and dissociation of the PD and PT of three or four pituitaries as previously described (52). Previous studies have demonstrated the validity of this method for producing a reliable hormone output in response to exogenous hormone releasing secretagogues in vitro (52, 56).

Both ELISA methods have been previously described (11, 57, 58). A rhVEGF<sub>165</sub>b-positive control was included in triplicate for the human VEGF-A ELISA, allowing calculation of VEGF-A<sub>total</sub> concentration to compensate for reduced VEGF-A<sub>xxx</sub>b affinity of ~42% as previously published (59). Prolactin was measured by RIA using purified ovine prolactin for standards. A linear relationship was detected when the measured hormone concentration (in nanograms per milliliter) was plotted against the concentration of diluted serum samples.

**Statistical Analysis.** In the BS and NBS cultures, a total of five separate experimental treatments were applied to PT cells, and nine experimental treatments were applied to the PD cells. For each treatment, six wells were assigned, and the experiments were repeated independently three times in both seasons with reproducible results. The reported values represent the mean ± SEM. The effects of season and experimental treatment and their interaction on the secretion of VEGF-A and prolactin from ovine primary pituitary cell cultures were examined by using ANOVA followed by Fisher's post hoc test. Because a season by treatment interaction was observed for each compound, separate ANOVAs were then used to examine the effects of experimental treatment within season. For all other variables, one-way ANOVA was applied. All data were confirmed to be normally distributed by D'Agostino and Pearson omnibus normality test. Data were considered to be statistically significant at  $P < 0.05$ ; however, wherever detected, smaller log value ( $P < 0.01$ ,  $P < 0.001$ ) probabilities are reported.

**ACKNOWLEDGMENTS.** The authors thank the National Hormone and Peptide Program and Dr. A. F. Parlow (University of California, Los Angeles) for prolactin radioimmunoassay standards, and Prof. A. S. McNeilly (Medical Research Council Human Reproductive Sciences Unit, Edinburgh, United Kingdom) for ovine prolactin antibody. This work was supported by the Biotechnology and Biological Sciences Research Council (D.J.T., J.C.-M.), the British Society for Neuroendocrinology (D.J.T.), and the British Heart Foundation (D.O.B.).

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