An updated view of hypothalamic–vascular–pituitary unit function and plasticity

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A 21st century perspective on hypothalamic–vascular–pituitary unit function

[Au: our journal style is to write 21st century in full as 'twenty first', which would take the title over the length limits for this type of article. We also use 'perspective' for a specific opinion-based article. To reduce the length of the title and avoid the use of perspective, I suggest the following title modification:

"An updated view of hypothalamic–vascular–pituitary unit function". OK?]

[Au: you use a mixture of hypothalmo and hypothalamic in the main text. I suggested consistently using on hypothalamic, changes throughout main text also OK?]

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Abstract [Au: edits OK?]

The discovery of novel functional adaptations of the hypothalamus and anterior pituitary gland for physiological regulation has transformed our understanding their interaction. The activity of a small number of hypothalamic neurons can control complex hormonal signalling, which is disconnected from a simple stimulus and subsequent hormone secretion relationship but is dependent on physiological status. The interrelationship of hypothalamic neurons and pituitary cells with the vasculature has an important role in determining the pattern of neurohormone exposure. Cell in the pituitary gland form networks with distinct organizational motifs that are related to the duration and pattern of output. These networds are modified in different physiological states and can persist after cessation of demand, which results in enhanced function **[Au: edit OK?]**. Consequently, the hypothalamus and pituitary can no longer be considered as having a simple stratified relationship: they form a tripartite system with the vasculature, which must function in concert for appropriate hypothalamic regulation of physiological processes, in particular reproduction **[Au: edit OK? I think it's important**

to mention reproduction in the abstract here]. An improved understanding of the mechanisms underlying these regulatory features has implications for current and future therapies that correct defects in the hypothalamic–pituitary axis. In addition, recapitulating proper network organization will be an important challenge for regenerative stem cell treatment.

[Au:For your information, H1 and H3 refer to the level of heading and will be removed before proofs are made. H1 subheadings can have max 38 characters inc spaces. H3 subheads can be of any length. Subheads have been edited to fit these limits, where indicated]

[Au: I think the Review would benefit from a figures introducing the basics of the concepts you discuss. I think this will be important to increase the impact of the paper, and for our less informed readers who are endocrinologists, but not particularly familiar with the neuroendocrine aspects. I have included the details of the figures you might wish to consider adding at the end of this document. I have also indicated in the main text where I think new figures might be useful additions. When you submit your revisions, please submit any new figures and I will work with our art editor to generate the first versions. We can revise these before making the final proof. If you wish to discuss these in greater detail before submitting, please do not hesitate to contact me]

[H1] Introduction [Au: I have moved the second section (originally called 'Hypothalamic–pituitary axes' of your original draft to here to serve as an introduction (the opening section is always titled 'Introduction' in our style guide). I think this text is more appropriate here and sets up the rest of the article nicely. Please also include the appropriate references in this section]

To maximize reproductive success, via the appropriate timing of ovulation, lactation or body growth, the output of several hypothalamic–pituitary axes are dramatically altered **[Au: edit OK?]**. These adaptive changes occur over differing time scales, with varying frequencies and levels of predictability **[Au: is there a general review you can cite here?]**. For example, the increase in growth hormone (GH) output at puberty is largely predictable **[Au: meaning the levels are predictable or simply that it occurs?]**. On a relatively short time scale **[Au: meaning measured in days?]**, the surge in luteinizing hormone (LH) secretion required for oestrus is an acute change that occurs regularly once every reproductive cycle and, in humans, continues for years in the absence of pregnancy. On a longer time scale **[Au: meaning months/years?]**, the increase in prolactin required for lactation is maintained for a variable time (which depends on when offspring are weaned) and recurs at each pregnancy, but is unpredictable before gestation **[Au: edit OK to avoid repetition of 'pregnancy'?]**. These large changes in output require modification of both hypothalamic and pituitary function, but are reversed after pregnancy, which likely reflects the fact that these processes need to be

repeated in subsequent pregnancies **[Au: edit OK is this what you?]**. A mechanistic understanding of these alterations in hypothalamic–pituitary function is fundamental to interpret and treat defects that lead to endocrine diseases **[Au: please be more specific here as to the diseases you mean in this context to set the scene for your review]**. In this Review, we will focus on three pituitary axes that have roles in driving changes in physiology; the gonadatropin, prolactin and growth hormone axes **[Au: addition OK?]**. The level of our understanding varies for each of these axes and the features that might serve as general principles will be highlighted in the text **[Au: edit OK?]**.

[H1] Beyond stimulus-secretion coupling **[Au: I think you could include a simple figure in this section on stimulus-secretion as an addition to BOX 1. And based on the summary figure you have already submitted. I think these concepts will work well in graphical form for our less informed readers]**

The speed of communication between the brain and peripheral tissues is highlighted by muscle contraction, which requires the transfer of electrical signals from axons via the neuromuscular junction **[Au: edit OK? Is this what you mean here?]**. This sequence of events, known as excitation-contraction coupling[1], takes <1 s in mammals and is highly plastic **[Au: please elaborate on what you mean by plastic in this instance]**. Similarly, in the hypothalamus just a few thousand neurons can also send signals to the periphery, in this case toward the median eminence (ME) via a specialized neurohaemal junction **[Au: edits OK?]**. Here, nerve terminal depolarization, either originating from the perikarya[2] or the terminal itself[3], allows the sufficiently rapid entry of calcium ions to trigger exocytosis of neurohormones towards the first loop of the portal fenestrated capillaries **[Au: please reference this statement here]**. This rapid (< 1 s) sequence of events was termed 'stimulus-secretion coupling' due to the clear similarities with excitation-contraction coupling[4,5]. Soon after release, neurohormones pervade the second loop of fenestrated capillaries within the downstream pituitary gland, before binding to cognate receptors on endocrine cells to induce pituitary hormone exocytosis through a second 'stimulus-secretion coupling' event[6-9]. **[Au: I think here might be a nice place to include a figure detailing these events for our less informed readers. A simple outline/schematic would be sufficient and that will set the scene for understanding the different axes later in the text]**

In the second half of the twentieth century (and building on Harris' work on the hypothalamus–pituitary axis **[Au: please reference this statement with a reference to**

Harris' work for our less informed readers]) the analogy between excitation-contraction and stimulus-secretion coupling was developed further **[Au: edit OK?]**[10]. However, important and fundamental differences exist between the two processes. Specifically, in the hypothalamus, endogenous **[Au: circadian]** rhythms exist and the time scale for pituitary hormone release is much longer (measured in minutes to several hours) **[Au: edit OK? Please reference this statement]**. Nevertheless, the analogy with neural control of locomotor activity led to a generally accepted model of hypothalamic regulation of pituitary function. Specifically, the excitation of specific hypothalamic neuron populations, determined by higher brain centres and peripheral feedback, is relayed as an unmodified series of signals to drive balanced pituitary hormone output **[Au: please reference this statement]**. The release of neurohormones and subsequent transportation and the effects on target cells were previously considered to be passive events in the regulation of pituitary hormone secretion, with only variation in the number of endocrine cells seeming to affect response levels[11,12]. Similarly, the alterations in gene expression and cell proliferation, which support maintenance of hormone output, were simply considered a correlated response to hypothalamic regulation of secretion **[Au: please reference this statement]**.

In the early twentyfirst century, a series of paradigm shifts in our understanding of the hypothalamic–pituitary system was established as a consequence of newly developed tools and techniques **[Au: such as?]** for use in genetically modified mice **[Au: please reference this statement with some examples]**. The use of these methods have shown that both the pituitary gland and portal system can no longer be considered as static structures simply responding to neurohormonal regulation (BOX 1) **[Au: edits OK to shorten sentence? I think the text box should be cited later in the text as more of a summary section]**. In addition, hypothalamic neuron function has been found to be more dynamic than initially thought, which might, rather than changes in excitation, contribute to modifications in its regulation of the pituitary under different physiological states **[Au: edit OK? Please reference this statement]**.

[H1] Gonadotroph axis

The reproductive system is critically dependent upon pulsatile secretion of gonadotrophin-releasing hormone (GnRH) and LH; however, the understanding of pulse generation has been hampered by the complexity of the regulatory mechanisms, many of which are lost in *in vitro* preparations **[Au: please include the references here also as examples]**. Investigators

working in the late 1980s using pituitary portal bleeding and microdialysis documented the pulsatile nature of GnRH release into the portal vasculature of the sheep, monkey and rat[25-31], and showed a strong correlation between GnRH and LH pulses[28,32,33]. However, the scattered distribution and relative paucity of GnRH cell bodies limited the investigation of the cellular events that lead to pulsatile secretion of LH *in vivo*. In the past few years, the development of optogenetic techniques in rats and mice and an ultra-sensitive ELISA capable of measuring LH levels in small whole blood samples[34] has enables investigators to dissect the GnRH neuron excitation parameters that generate LH pulses[35]. In these studies, the stimulation of just 60 GnRH neurons can trigger short-lived increases in LH secretion that resemble endogenous pulses **[Au: Edits OK? please reference this statement]**. Given the critical importance of GnRH neurons to the survival of all mammalian species, a degree of functional redundancy within this cell population is expected. Indeed, activating ~5% of the GnRH hypophysiotropic neurons seems to be sufficient to generate an LH pulse **[Au: please reference this statement]**. This finding is consistent with studies in which just 10% of the GnRH neuron population is sufficient to maintain pulsatile LH secretion[36,37]. When the timing and frequency of stimulation is varied a brief (2 min) optogenetic stimulation at high frequency (10 Hz) evokes an LH pulse, whereas shorter periods and lower frequencies cannot elicit LH output that resembles endogenous pulses[35]. **[Au: edit OK?]** This finding was also the case for a bursting pattern of stimulation **[Au: meaning of LH secretion? How is a bursting pattern defined in this context?]**, which had been assumed to be effective for pulse generation and the focus of many previous studies[38] **[Au: meaning that this mechanism was the only one thought to be necessary of LH output?]**. Whether such a stimulatory signal exists *in situ* **[Au: meaning within the organism?]** and where its origin might be is unknown, although a 'GnRH pulse driver' might be located in the mediobasal–hypothalamus, specifically at the level of neurons co-expressing kisspeptin, neurokinin B, as well as dynorphin A (so-called 'KNDy' neurons)[39,40]. **[Au: Edits OK? perhaps you can expand on that specific evidence here for clarify?]**

Pulsatile secretion of GnRH requires synchronization **[Au: of the secretion and/or pulsatilty itself? What do you mean in this context?]** within the GnRH neuron population. While the cell bodies of GnRH neurons are scattered throughout the basal forebrain, their projections have dendrodendritic bundling, that is they share synapses **[Au: edit OK, is this what you mean?]**[41], and become highly concentrated around the ME **[Au: please reference this statement]**. Fascinatingly, these projections simultaneously receive and integrate synaptic

inputs — they possess both axonal and dendritic characteristics, leading to their description as 'dendrons', before finally acquiring an axonal morphology within the ME and ramifying into numerous terminals that appose blood vessels[42]. **[Au: I think this could be represented in the Figure also. It will then also give context to the vasculature aspect of the article]** Dendrons might be an ideal location for putative afferent axons to modulate the excitability of multiple GnRH neuron dendrites, and for multiple GnRH neurons to align their firing pattern, which thereby provides a potential mechanism for their synchronized activity directly in the mediobasal hypothalamus **[Au: is this your opinion?]**. An additional source of pulse synchronization is in the ME, where hypophysiotropic GnRH neurons terminate within the external zone close to endothelial cells of the portal vasculature[43]. Endothelial cells in the ME might modulate GnRH release through nitric oxide secretion (which has been reviewed elsewhere[44]) **[Au: edit OK? I would include some of the original reference here also to reduce the nu,ber of other reviews cited]**. At the ME, nitric oxide is spontaneously released from an endothelial source and follows a pulsatile and cyclic pattern of secretion[45], and inhibition of nitric oxide synthesis **[Au: by local do you mean specifically in the ME or a wider area?]** can disrupt reproductive cyclicity[46]. Conversely, in the GnRH neuron perikarya, basal nitric oxide synthase activity might provide the tonic inhibition of the GnRH neural system required to maintain nadir levels of LH **[Au: edit OK?]** [47]. **[Au: this mechanism to be represented in the figure also?]**

Once released into the ME, the transport of GnRH to the pituitary, and the pattern of gonadotroph exposure to the neurohormone, have been largely assumed to represent a simple linear process **[Au: please reference this statement]**. However, the use of fluorescent tracking using 4 kDa dextran, which mimics the size of most hypothalamic neurohormones, has shown that the diffusion processes, both at the level of the ME and the pituitary capillaries, are complex and non-linear [7]. **[Au: edit OK?]** Consequently, the portal vessel network might function as a 'physical integrator', enabling neurohormones to be transferred from the ME to the gonadotroph within a few seconds **[Au: can you cite a reference here?]**. Once in the blood stream, the moderately rapid clearance rate **[Au: can you define this rate here?]** of LH underlies **[Au: meaning it generates it?]** the specific asymmetric pulse shape of this hormone, which is characterized by a fast increase immediately followed by a slower decrease[34]. Importantly, a faithful delivery of the pulsatile pattern of GnRH secretion to the pituitary is crucial for gonadotroph function[48] **[Au: please include some of the original key references for this finding]**. For example, high GnRH pulse frequencies (>1 pulse per h)

activate LH production, whereas low frequencies (<1 pulse per 2–3 h) preferentially induce follicle-stimulating hormone (FSH) synthesis and release[49]. Overall, the intricate relationships between pulsatile GnRH release, secretory competency of the pituitary gonadotrophs and regulatory mechanisms within the vasculature, generate the rhythmic fluctuations in LH secretion.

[H3] GnRH and LH surge generation

The GnRH/LH surge mechanism is sex specific and normally occurs only in women [50-52] **[Au: Please include the original references and only one Review here; it might be interesting to add a note about when it might occur in men]**. During the oestrous cycle, increasing concentrations of plasma oestrogen alter feedback to the GnRH neuronal afferent networks and gonadotrophs from negative to positive to induce the gonadotrophin surge **[Au: please reference this statement]**. That the oestrogen-responsive kisspeptin neurons in the rostral periventricular area of the third ventricle have a critical role in enabling ovulation in rodents by activating GnRH neurons is now well accepted [53]. Importantly, the relative contribution of the hypothalamic and pituitary levels to the oestrogen-induced gonadotropin surge seems to be species-dependent, with the latter the predominant mechanism in human and non-human primates[54]. In the female sheep, the GnRH surge is composed of high-frequency pulsatile events superimposed on a constantly elevated level of GnRH release, although whether the surge is driven by a fundamentally altered pattern of GnRH secretion[55], or by a simple increase in the frequency of pulsatile secretion is unclear **[Au: edit OK?]**[56]. This huge increase in GnRH secretion continues for a period of 24 h, considerably longer that the duration of the LH surge it induces, before returning to a strictly episodic pattern of release[31,55,57]. The firing pattern of GnRH neurons needed to generate the GnRH/LH surge is unknown. However, that the prolonged firing of an increased number of GnRH neurons is required for the secretion of surge levels, compared with that required for a pulse, is a reasonable assumption **[Au: edit OK? Have I retained your meaning here?]**. Indeed, mice with 10% of the normal GnRH neuronal content failed to ovulate **[Au: meaning 90% were ablated? How so?]**, but cyclicity was restored when approximately 30% of the GnRH population was present [36,37]. **[Au: please include a little more detail of how these studies were conducted]**

In addition to the putative change in GnRH population electrical activity, anatomical changes are found within the external zone of the ME where GnRH nerve terminals are ensheathed by tanycytes[58,59]. The cellular conformation changes with fluctuating oestrogen profiles throughout the oestrous cycle. For example. in rats, semaphorin-7a-dependent structural

remodelling of tanycytes occurs during the preovulatory surge, resulting in release of the engulfed axons and direct access of GnRH nerve terminals to the portal vasculature **[Au: please reference this statement also]**. By contrast, fenestrated endothelial cells of the hypothalamic–hypophyseal portal vessels release semaphorin-3A, which is thought to induce GnRH neuron axonal growth and sprouting within the ME as a function of the oestrous cycle[60]. **[Au: also data from rats?]** These mechanisms are likely to enable the generation of high concentrations of GnRH, which evoke the GnRH/LH surge, to be released into the pituitary portal circulation[61-63].

Within the pituitary, the distinct network organization of gonadotrophs[14] and their large scale reorganization during puberty[15] suggests that homotypic cell organisation **[Au: meaning the cells reorganize according to their type? Please expand on what you mean here. Can this be included in the figure?]** might have a functional role **[Au: in puberty itself?]**. However, this reorganisation **[Au: edit OK? Is this what you mean here?]** has not been studied in detail in the other pituitary axes to date. Although the dynamic gonadotroph responses at the time of the proestrous surge have not yet been described *in vivo*, static snapshots **[Au: meaning 'individual readings'?]** in rats and sheep suggest that changes occur in gonadotrophin subunit expression, granule distribution and GnRH receptor abundance[64,65]. **[Au: edit OK?]** However, mRNA sequencing of the anterior pituitary glands from women **[Au: of what age?]** reveal that genes regulating **[Au: gonadatroph?]** secretion, blood pressure and cell adhesion were all enriched during proestrus[66]. Likewise, immortalized cell lines and cells in pituitary slices both extend cellular processes and increase their cellular movement at **[Au: stages that replicate puberty?]** puberty[15] and following GnRH stimulation[67]. These findings suggest that changes in the relationship of the gonadotroph network with the vasculature might modify the secretory response of gonadotrophs[68,69]. **[Au: edit OK?]**

[H3] Clinical relevance

The mechanisms that underlie both pulsatile secretion and surge generation of LH have important implications for the treatment of infertility in women **[Au: edit OK?]**. For example, polycystic ovarian syndrome (PCOS), the most common anovulatory cause of infertility[70] and affects >100 million women worldwide, is associated with a dysregulation of the normal pattern of LH secretion **[Au: please reference the latter part of this statement also]**. Whether the origin of this multi-factorial disorder is at the level of the hypothalamic–pituitary axis is unknown[71], but PCOS is characterized by increases in GnRH pulse frequency **[Au: of**

how much? Can this be quantified?] and sensitivity of the pituitary gland to the neurohormone[72,73]. Consequently, potential interventions that modify the dynamics of GnRH output, its transport to the ME or its actions in the pituitary might have implications for the treatment of PCOS **[Au: is this opinion?]**. This is also the case for congenital hypogonadotropic hypogonadism **[Au: we avoid using two letter abbreviations where possible. HH removed OK?]**, which results from a pituitary or a hypothalamic defect with or without anosmia **[Au: please reference this statement for clarity also]**. Several novel gene mutations that are associated with this disorder have been identified, including those encoding neuropeptides **[Au: such as?]**, transcription factors **[Au: such as?]** and G-protein coupled receptors**[Au: such as?] [Au: please give examples here]**[74]. To induce female fertility, hypogonadotropic hypogonadism of pituitary origin can be reversed by subcutaneous injections of FSH followed by human chorionic gonadotrophin or LH to trigger ovulation **[Au: please reference this statement]**. Conversely, hypogonadotropic hypogonadism of hypothalamic origin can be treated using GnRH pumps to restore pituitary hormone secretion **[Au: please reference this statement]**. Pulsatile GnRH has the advantage of decreasing the risk of multiple pregnancy and ovarian hyperstimulation syndrome[75]. In both situations, the pulsatility of GnRH or the rhythmic secretion of FSH and/or **[Au: OK?]** LH is required to obtain sufficient follicular maturation and proper ovulation[76]. Advances in understanding of GnRH secretion and its interactions with LH are essential for designing novel, and indeed modifying existing, therapies for hypogonadotropic hypogonadism. For example, some patients **[Au: roughly how many? Is there a % you can include here?]** with this disease, who are yet to undergo treatment, have transient phases of normal fertility[77] **[Au: edit OK?]**. The underlying mechanisms and relevant therapeutic interventions to maintain this phenomenon might be elucidated by further investigation of pulsatility and rhythmicity **[Au: edit OK?]**.

[H1] The prolactin axis

The prolactin axis is unique amoung the pituitary hormonal systems, as in men and women who have not previously had sexual intercourse, it can be considered a system primed for activation by tonically inhibited by hypothalamic dopamine[78] **[Au: edit OK? Is this what you mean?]**. **[Au: 'In this situation'?]** Low concentrations **[Au: can this be quantified?]** of circulating prolactin are maintained by short-loop feedback, with prolactin receptor-mediated stimulation of dopamine neuron firing rate leading to an increase in catecholamine

production[79] and output[2]. The timescale of the secretory response for prolactin (~10–20 min) requires the coordination of multiple neurons **[Au: please reference this statement]**. Specifically, tuberoinfundibular dopamine neurons undergo long-term coordinated changes in firing rate, which correlate with episodic secretion **[Au: of prolactin?]** in mice[2]. **[Au: please expand on this finding. How do you mean long-term and what are the changes in the firing rate? I think you need to include a little more explanation here]** Gap junctions and local dendritic dopamine release have been proposed to mediate this activity[80,81], and integration of single cell firing rates seems to be involved in the generation of longer DA **[Au: DA meaning dopamine?]** release output events (N. Romano and P. Mollard, unpublished data).

Variations in prolactin output occur in virgin female rats as a surge at proestrus, which coincides with that of LH[82]. Prolactin also increases following vaginal stimulation of rodents **[Au: meaning it is true in mice and rats? Or has this only been shown in rats?]** as twice daily surges[83]. These surges are coordinated by signals from the suprachiasmatic nucleus[83], most likely through the actions of vasoactive-intestinal peptide[84]. At the level of the pituitary, lactotrophs form a network of honeycomb motifs that allow the congregation of cells along the fine pituitary capillary network[85]. **[Au: can this be represented in a figure summarising the Prolactin axis?]** This organization supports low levels of cell-cell coordination[16], with a small proportion **[Au: how many? Can this be defined?]** of cells acting as coordinating nodes by functionally connecting distant ensembles **[Au: please reference this latter part of the statement]**. In addition to synchronizing Ca^{2+} activity, cellular organization also mediates the coordination of gene transcription[86,87], with gap junction signalling enabling local correlation of bursts of transcriptional activity that are otherwise randomly timed **[Au: please reference this latter part of the statement]**. This mechanism resembles quorum-sensing where apparently random systems display complex activity as long as the components (in this case the cells) can interact, and might contribute to hormone gene expression and cell proliferation[87,88,89]. **[Au: edit OK?]** How gap junctions might orchestrate this mechanism remains unknown; however, regenerative Ca^{2+} waves and Ca^{2+}-regulation (*e.g.* through NFAT) could be one possibility[90]. **[Au: I think you need to expand on this highlighted (in grey) statement — e.g. why specifically could it be a possibility — or remove it altogether if this is too speculative]**

[H3] Increased prolactin output during lactation

The long-term requirement for large increases of circulating prolactin in lactation is associated with a decrease in dopamine output, which begins in late pregnancy[91] and is coincident with a surge of pituitary prolactin secretion **[Au: please reference this latter statement – 91 also? Edit OK to simplify sentence and avoid repetition?]**. The dopamine tone needs to be strongly decreased throughout lactation to enable the necessary increase in circulating prolactin, and is mediated by a decrease in phosphorylation of tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis[91]. This mechanism is not the result of a reduced feedback of prolactin on dopamine neurons, which remain electrically responsive at the level of the cell body, but rather, neuronal firing becomes uncoupled from dopamine secretion[2]. **[Au: edit OK? have I retained your meaning here?s]** Remarkably, the reduction in dopamine tone is accompanied by the production of opioids[92,93], which might enable these neurons to stimulate prolactin secretion **[Au: is this latter statement an opinion?]**.

In concert with changes in the hypothalamic inhibition of prolactin secretion, substantial alterations occur in the pituitary to support the 10–50-fold increase in prolactin secretion that is required for milk production in mammals[93] **[Au: edit OK?]**. In humans and rats, this **[Au: milk production?]** is generally accompanied by proliferations of lactotrophs, with some evidence of hypertrophy **[Au: of the lactrotrophs?]**, although these results **[Au: meaning the hypertrophy results?]** are based on 2D histological studies **[Au: edit OK?]**[94]. By contrast, in lineage tracing and FACS studies in mice, lactotrophs become hypertrophied during lactation and increase their volume threefold rather than simply increasing in number **[Au: edit OK?]**[95]. Other investigators have confirmed these findings, and also showed that the lactotroph network *in situ* becomes highly-connected during lactation, which is associated with the strength of the suckling stimulus[16]. **[Au: edit OK?]** This increase in structural connectivity leads to a substantial increase **[Au: of how much?]** in the proportion of the subpopulation of lactotrophs that function as coordinating nodes and orchestrate increased output of prolactin **[Au: please reference this statement]**.

[H3] Memory of prolactin demand after weaning ***[Au: edit OK?]***

At weaning **[Au: edit OK? I think the term weaning is well-enough understood to omit an explanation]**, a rapid decrease in prolactin secretion occurs as a result of a return of dopamine inhibition[96]. In rodents[97,98] and humans[99] basal prolactin secretion is reduced below that of virgin animals, which might reflect an enhanced pituitary response to dopamine inhibition[100]. Strikingly, and despite this reduction in basal prolactin secretion, lactotrophs

remain enlarged and well-connected **[Au: with each other? Or generally within the network? Please clarify here]** at both the structural and functional levels[16], with an increased proportion of cells acting as nodes **[Au: what is the proportion? What does it change from and to? What is the specific function of the nodes at this stage? Possibly to be included in a new figure on the prolactin axis]**, which persists for many months after lactation has ceased **[Au: edit OK, is this what you mean? please reference this statement]**. Such hard-wiring or 'memory' of previous stimuli, which was previously thought to only exist for neurons and immune cells, leads to a lactotroph network behaviour **[Au: what do you mean specifically by 'lactotroph network behaviours' in this instance?]** during subsequent lactations **[Au: meaning after subsequent births?]**, which drives even higher concentrations of prolactin[16]. This mechanism is independent of reproductive experience *per se*, since it can be prevented by reducing the suckling demand **[Au: please reference this statement]**.

[H3] Clinical relevance

The dysregulation of the prolactin axis, owing to either pituitary adenomas[101] or as an adverse effect of treatment with antipsychotic drugs[102], leads to impaired fertility. Hyperprolactinaemia is the second most common cause of infertility in women after PCOS **[Au: please reference this statement; how many individuals does hyperprolactinaemia affect?]**. A clear understanding of the interactions that lead to altered dopamine output and the response of the pituitary might help to identify novel treatment strategies for this disease. For example, further understanding the regulation of prolactin release by dopamine might enable the reduction in the doses of commonly-used dopamine receptor agonists, as their safety in the treatment of prolactinomas has been questioned[103]. **[Au: why so? Please include a little extra detail]** In rodent studies, prolactin seems to affect multiple neuroendocrine axes **[Au: such as? Please include the axes here for clarity]** [77], and these warrant further study to determine the potential effects of its over-secretion in humans. For example, hyperprolactinaemia might lead to changes in GnRH neuron activity via interactions with the GPR54/kisspeptin pathway in mice[104] and GnRH pulsatility has been reinstated in mice with physiological hyperprolactinaemia by administration of kisspeptin[105]. **[Au: edit OK?]** By contrast, in studies using sheep with a lower prolactin dose **[Au: lower dose than what? And how much lower?],** no effects on hypothalamic kisspeptin expression have been seen **[Au: in response to this treatment/administration please clarify here? edit OK?]**[106]. An improved understanding of these pathways could aid the development of treatments for women with

hyperprolactinaemia that is resistant to dopamine agonists **[Au: please provide a reference for this dopamine agonist-resistant hyperprolactinaemia]**.

[H1] The GH axis **[Au: suggest a new figure for this section including the organisation and plasticity of the GH axis]**

In humans and animals in which it can be measured **[Au: such as?]**, pulsatile GH output is present from birth[107,108,109]. However, the output is markedly increased at puberty when sexually-dimorphic body growth occurs[110]. **[Au: edit OK?]** Since the discoveries of GH releasing hormone (GHRH) and somatostatin that control GH secretion from pituitary somatotrophs[111-113], a remarkable advancement our understanding of GH pulse generation during critical physiological windows has taken place **[Au: edit OK?]**.

[H3] Pulsatile GHRH output

Using genetically-modified mouse models with GHRH neurons marked with green fluorescent protein[114], several investigators have defined the mechanisms that underlie pulse generation using *ex vivo* slices of brain **[Au: edit OK?]**. Before puberty, GHRH neurons are excitable neuroendocrine neurons with complex synaptic inputs[115]. These early stages of hypothalamic development ensure appropriate regulation of the somatotroph axis, as in the Ames dwarf mice in which loss of GH leads to a compensatory increase in GHRH cell number[116], **[Au: edit OK?]** and programming effects of steroids on GHRH cell number and gene expression[117] **[Au: please expand on this latter statement. What specifically do you mean by 'programming' and how does the cell number and gene expression change?]**. Modification of these synaptic inputs and electrical properties over the first 6 postnatal weeks correlates with and likely drives, at least in part, increased pituitary GH output and sexual dimorphism[118]. In all these studies, the intrinsic hourly rhythms of GHRH neuronal activity predicted by simulation studies of *in vivo* GH pulsatility have not been indentified[115,119]. However, somatostatin can generate GHRH neuron pulsatile output by delaying oscillations of action potential firing via a recurring inhibition of inhibitory GABAergic interneurons (that is, inhibition of inhibition)[119]. **[Au: edit OK? Have I retained your meaning here?]** Consequently, somatostatin can both acutely inhibit the excitability of GHRH neurons and also promote their patterned output together with more sustained GHRH neuron stimulation in response to other stimuli in the brain (for example, acetylcholine[118]) and peripheral tissues (such as, ghrelin) [113]. **[Au: edit OK?] [Au: can these processes be represented in the a figure on the GH axis? E.g. to highlight the plasticity of these neurons during puberty?]**

[H3] Modification of pituitary somatotroph output

No full description of the *in vivo* dynamics of GHRH and somatostatin neurons and their regulation of pituitary somatotrophs exist **[Au: edit OK?]**. This event can be viewed as a three-step process: delivery of neurohormone to target cells; cellular secretory responses to regulation; and entry of pituitary hormone into the peripheral circulation **[Au: please reference this statement. Or is this based on opinion?]**. *In vivo* imaging of the mouse portal system and somatotroph network have provided insights into the first step in this process and the role of the vasculature in shaping the pattern of exposure of the pituitary to hypothalamic neuropeptides[7]. Delivery of neuropeptides such as GHRH to the somatotroph network, which extends throughout the pituitary gland, follows specific vascular/capillary routes and results in specific temporal patterned regulation rather than a homogenous 'immersion' **[Au: by 'immersion' meaning the whole pituitary exposure to the secretagogue?]** of the pituitary parenchyma in secretagogue **[Au: please reference this statement]**. In addition, the initial stimulation by GHRH evokes a coordinated enhancement of oxygen to the stimulated somatotroph network via increased capillary blood flow, which provides fuel for energy-depleting secretory responses **[Au: please reference this statement]**. Indeed, the GH **[Au: neuronal?]** network is spatially organized versus the vasculature **[Au: Do you mean that the GH network is organized in the same spatial manner as the vasculature? Do you mean all of the vasculature in this instance? please reference this statement]**, which suggests an important role of local oxygen regulation on GH release and that this may also be the case for other pituitary axes **[Au: is there any evidence to support this highlighted statement? If not I suggest you remove it]**. The second step in pituitary regulation has been characterized using *ex vivo* data from acute pituitary slices where the somatotroph network organization is preserved, in which the homotypic network-mediated coordination of stimulation, triggering long-lasting GH secretion[20]. These studies have also shown that network organization is likely to have a major role in the increased GH output at puberty. In particular, the GH network undergoes large changes in the volume to surface ratio **[Au: of what specifically?]** that coincides with the onset of puberty, before gradually returning to normal pre-pubertal levels by **[Au: postnatal?]** day 100 in mice **[Au: please reference this statement]**. Such changes correlate with sexually dimorphic patterns of output[13]. **[Au: please expand on this statement, what do you mean precisely here?]** Sex steroid manipulation also leads to rapid and dramatic increases in cell motility that remodels the network organization[120] **[Au: please can you expand on the details of this finding here]**. **[Sentences rearranged to improve**

flow OK? Have I retained you meaning here?] These findings highlight the importance of somatotroph network organization and its plasticity in the generation of pituitary somatotroph output. The vasculature also has an important role in somatotroph output, where *in vivo* imaging shows that capture of secreted GH is a controlled event where the perivascular space acts as a gate-keeper for hormone entry into the capillary lumen[120]. The relationship between neuronal network organization and the vasculature in the pituitary is, therefore, central to the delivery of incoming hypothalamic signals, and the build-up of GH pulses within capillaries.

[H3] Clinical relevance

GH deficiency resulting from congenital defects or acquired following traumatic brain injury, pituitary tumours or cranial irradiation[121,122], is commonly treated with a daily subcutaneous dose of recombinant GH in childhood to increase growth rate **[Au: please reference this latter statement]**. However, considerable uncertainty exists regarding the optimal dosage or regime, and current treatments **[Au: specifically GH injections?]** do not fully recapitulate the physiological pattern of GH secretion **[Au: please reference this statement]**. One potential therapy is repopulation of the pituitary with stem cells, which has been investigated in the mouse **[Au: addition OK? was this approach successful?]**[123]. However, such approaches would require the recapitulation of the normal cellular organization to achieve normal pulsatility and homeostatic regulation **[Au: edit OK?]**.

Patients with acromegaly, which results from a GH secreting pituitary adenoma, frequently have glycaemic disorders: a lack of GH pulsatility modifies lipolysis, whereas overall GH hypersecretion can induce insulin resistance[124]. Consequently, an improved understanding of the mechanisms that determine the pattern of GH output might help to define new therapeutic options for dyslipidaemia or diabetes mellitus. In addition, GH pulsatilty also has an important role in a subgroup of patients who have clinical acromegaly with increased insulin-like growth factor 1 **[Au: abbreviation used once removed OK?]**, but unaltered mean 24-h GH concentration compared with healthy controls[125,126]. Altered GH pulsatility might explain the clinical presentation of this sub-group of patients, and our new understanding of the mechanisms underlying patterning of pituitary output might explain the abnormal GH axis function in these individuals and warrants further investigation **[Au: edit OK?]**.

[H1] Conclusions

The examples in this Review provide new insights into regulation of three hypothalamic–pituitary axes and demonstrate that these mechanisms are not a simple relay of stimulus-

secretion coupled events **[Au: edit OK?]**. The disconnection or modulation of hypothalamic excitation and neurohormone release, and an active role of the vasculature and pituitary in the network-mediated modification of responses, demonstrates that this view of the hypothalamic–pituitary system is over-simplistic (FIG. 1). **[Au: we avoid first citing figures in the conclusions, but I think the addition of new figures as suggested above we negate the need for this Figure here and these points can be introduced earlier. You can then refer back to the ealier figures here in the conclusions] [Au: sentence removed here to avoid repetition]** Given that the hypothalamic arcuate nucleus contains no more than a few thousand parvocellular neurons, the rapid development of techniques for interrogating neuronal function should enable the characterization of this structure's regulation and output. Such studies will be invaluable for the deeper understanding of mammalian physiology, as this region controls a much larger panel of known body functions than any other brain region. In the vasculature and pituitary gland, many of the mediators of functional modulation remain unknown. Although loss of Prop1, which is essential for pituitary hormonal cell development, leads to loss of organ vascularization[127], studies of the processes leading to normal vascularization and its modification have not been reported to date. Similarly, hypothalamic and steroid factors have been shown to regulate cell network organization[19,20,120] but the underlying mechanisms and mediating factors are yet to be identified. The alteration of the gonadotroph network in mice with a developmental block of corticotroph terminal differentiation[14], suggests that the mechanisms will be complex and involve interaction between multiple pituitary cell types. **[Au: I suggest moving this information (highlighted in grey) into the preceding sections where appropriate. We try to avoid including new information like this in the conclusions, which should be for summing up the key points. These findings might have more impact in the earlier sections]**

The regulation of pulsatile pituitary secretion must now be considered as an integration of hypothalamic, vasculature and pituitary regulation, which has further implications for the understanding of disease **[Au: edit OK?]**. For example, the identification of kisspeptin has provided an exciting new target for the treatment of infertility[128]. The uncoupling of neuronal excitation and hormone output also has deeper implications such as in the case of ageing **[Au: addition OK? Is this what you mean here?]**. For example, the reduction in GH output with age might be due to a failure of neurohormone secretion from GHRH neurons that are excited under normal conditions **[Au: edit OK?]**[129]. The human pituitary gland can be accessed by transphenoidal surgery, which makes this structure ideal target organ for regenerative therapy

[Au: please reference this statement]. The pituitary neuronal networks and their relationship with the vasculature must be considered for such therapy and the microenvironment clearly has an important role in the regulation of the pituitary gland, which might also affect the development of tumours **[Au: edit OK?]** [130]. Finally, as pituitary networks are sensitive to peripheral regulation and their modification can persist for extended periods, they are a potential target for endocrine disrupting chemicals (EDCs). Indeed, the identification of EDC **[Au: bisphenol A?]**-mediated changes in expression of *ICAM5* **[Au: which specifically?]** in the pituitary[131] has led us to speculate that some EDC effects might be mediated by changes in pituitary organization[132]. These possibilities require further investigation for the understanding of both the aetiology and treatment of diseases associated with pituitary hormones **[Au: edits OK?]**.

[Au: Please re-reference to reflect editing changes (your citation manager should do this automatically on your machine) and put into our format– there should be an option to display bibliography in the style of 'Nature Reviews' in your reference manager program. Specifically, please remove the doi designations from the end of the references, which are not needed, unless an article has yet to be assigned a full page number and volume reference]

Example reference styles

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If a reference has six or more authors, only the first author should be listed followed by *et al*.

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Competing interests statement

The authors declare no competing interests

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Author contributions [Au: OK?]

The authors contributed equally to all aspects of the preparation of this article

Key points [Au: edits OK?]

- The activity of hypothalamic neurons is modified by inputs leading to heterogeneous activity; a small proportion of the total population can drive pituitary hormone pulsatility
- Neurohormone output can vary following neuron excitation according to physiological status, which might also lead to declining neuroendocrine output with age
- The release of hypothalamic factors into the blood is modified by alterations in the juxtaposition of nerve terminals with the vasculature and tanycytes in the median eminence
- Cells in the pituitary gland form homotypic networks, the organization of these relationships with the vasculature are distinct for each endocrine axis, which modifies responses to regulatory factors and patterns of output in response to demand **[Au: edit OK?]**
- Reorganisation of the pituitary network can store long-term memories of increased output and learn to increase function on repeated challenge
- Understanding the importance of coordinated hypothalamic–vasculature–pituitary function provides new understanding of a range of endocrine axes defects and targets for novel therapies

[Au: a list of suggested Figures and content:

Figure 1 – General introduction of the relationship between the 3rd ventricle, median eminence, portal vessels, vasculature and pituitary. We have a range of in-house options that can be adapted for this purpose. – I can send you an example or two if you wish

Figure 2 – The gondadotroph axis – to include the organisation of the GnRH/LH network in the hypothalamic/pituitary/vasculature and with factors that might influence (e.g. NO). Might also include the homotypic organisation at puberty. E.g. a 'before' and 'after' panel

Figure 3 – The prolactin axis – could include a representation of the lactotrophs and their development into networks – e.g. an expansion of what is represented in the bottom left of current Figure 1.

Figure 4 – The GH axis – to include a graphical view of this axis and could include how this axis is modified to develop the pulsatility or the modification of pituitary somatotroph output

If simpler, perhaps proposed Figures 2, 3 and 4 could be combined into a single, multi-panel figure if you wish?

Figure 5 – summary of our current understanding – based on your submitted figure 1, although some of these elements can be included in the above figure

Figure 1 | Regulation of the hypothalamic-pituitary axes. In the past decade, a more complex relationship between the hypothalamus and pituitary than previously appreciated has emerged. **a**) At the level of the parvocellular neurons, complex inputs modify the excitation of neurons, which can vary coupling with neurohormone output at terminals of the median eminence (ME) through modification of intracellular pathways. **b**) Alterations in tanycyte ensheathment and the anatomical location of neuron terminals modify their interactions with the vasculature, changing the dynamics of neurohormone release. **c**) In the pituitary gland, changes in blood flow in the portal circulation alter the pattern of exposure of pituitary cells to neurohormone and nutrient supply to facilitate secretion. **d**) Cells of the pituitary gland are organised into intermingled networks with distinct morphological features, which can be altered to meet physiological demand, and relationships with the vasculature.

[Au: I have made a number of edits to the text in this box to adhere to journal style and the overall flow of the article. However, I think some of these concepts could be represented in the suggested figures]

Box 1 | Organization of pituitary cells into homotypic networks

During the past decade, we and others have shown that the pituitary somatotroph[13], corticotroph[14], gonadotroph[14,15] and lactotroph[16] lineages form 3D organized cell networks (reviewed in[17,18]) with the following features:

- *The lineages have distinct developmental programmes*
    - Placement of each endocrine cell network occurs at distinct stages of pituitary organogenesis, before expansion in early post-natal life **[Au: please include the references here]**.
- *The networks have distinct motifs that might relate to function*.
    - For example, the organization of somatotrophs as clusters linked with strands[13], while lactotrophs form a honeycomb structure[16]. **[Au: how do these relate to function specifically? Or is this just an association?]**
- *Hypothalamic factors generate network motifs*
    - For example, the loss of growth hormone-releasing hormone (GHRH) itself leads to isolated somatotrophs, whereas somatotroph ablation with intact GHRH stimulation results in clusters of cells that are isolated from each other[19].
- *The pituitary networks have functional relevance.*
    - Critical roles have been described for pituitary networks in the integration, amplification and propagation of the neurohaemal hypothalamic signals that arrive via the median eminence. For example, the male somatotroph network responds to GHRH input with large, coordinated and oscillatory Ca^{2+} rises that outlast stimulus to drive large excursions in hormone secretion[20].
- *Endocrine and non-endocrine cells interact to form homotypic networks* ***[Au: edit OK? Is this what you mean here?]***.
    - For example, gap-junctions mediate coupling **[Au: of what cells specifically?]** with folliculostellate cells[16,21], paracrine and autocrine interactions[17,22] as well as the relationship between networks and the vasculature[7,23,24]. These interactions might help to alter **[Au: somatotroph or simply hormone?]** output. The distinct network motifs of lactotrophs are aligned with the fine pituitary capillary network, while somatotrophs form strands and clusters along the same vessels[17]. Similarly, gonadotrophs are in close approximation to one or more blood vessels via their protrusions[15], whereas the corticotroph network has a looser arrangement [14]. **[Au: meaning these differences will shape how the neurohormones are released e.g. in terms of timing?]**

Author biographies [Au: edits OK?]

Paul Le Tissier is a Senior lecturer in the Centre for Integrative Physiology, University of Edinburgh, UK. His primary research interests are the understanding of hypothalamic regulation of the anterior pituitary gland, how this mechanism maintains and alters the function of the different cell populations to ensure appropriate output throughout life and modelling how dysregulation leads to pathology.

Pauline Campos has recently become a postdoctoral fellow in the laboratory of Patrice Mollard, at the Institute of Functional Genomics of Montpellier, France. She completed her PhD in the Center for Neuroendocrinology at the University of Otago, New Zealand, and has been focused on developing cutting-edge tools to study the electrical activity of hypothalamic neurons underlying pituitary hormone pulsatility. Her current work aims at understanding the central controls of thyroid function in conscious animals

Chrystel Lafont is an INSERM engineer in the Institute of Functional Genomics of Montpellier, France. She is the technical leader of a small animal *in vivo* imaging facility that develops cellular *in vivo* imaging techniques. Her research interests look at the role of the vasculature and pituitary cell networks in the build-up of hormone pulses.

Nicola Romanò is a postdoctoral fellow at the University of Edinburgh, UK. His main interest is the study of the cellular and systemic mechanisms of pulsatile hormone secretion in neuroendocrine axes, and how they correlate and influence physiological function. His research interests span from analysis of activity patterns of hypothalamic neurons to structural and functional analysis of pituitary cell networks, such as, the spatio-temporal analysis of cell activity.

David Hodson is a Senior Birmingham Fellow, Diabetes UK–RD Lawrence Fellow and European Foundation for the Study of Diabetes/Novo Nordisk Rising Star Fellow. He studied Veterinary Medicine at the University of Bristol, UK, before becoming a group leader at Imperial College London and latterly at the University of Birmingham, UK. His laboratory is developing innovative optical methodologies that enable the mapping and manipulation of endocrine cells directly within their tissue context.

Patrice Mollard is the head of the Department of Physiology and CNRS Research Director at the Institute of Functional Genomics of Montpellier, France. Dr Mollard's laboratory pioneered the study of large-scale functional organization of endocrine cell types within the pituitary gland in the early 2000s. His research is now exploring how pituitary cell networks function together with hypothalamic inputs and vasculature in health and disease.