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The Processes Of Anterior Pituitary Hormone Pulse

Generation

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1 **Abstract**

2 More than 60 years ago, Geoffrey Harris described his “Neurohumoral theory” in which the
3 regulation of pituitary hormone secretion was a “simple” hierarchal relationship, with the
4 hypothalamus as the controller. In models based on this theory, the electrical activity of
5 hypothalamic neurons determines the release of hypophysiotropic hormones into the portal
6 circulation and the pituitary simply responds with secretion of a pulse of hormone into the
7 bloodstream. The development of methodologies allowing monitoring of the activities of
8 members of the hypothalamic-vascular-pituitary unit is increasingly allowing dissection of the
9 mechanisms generating hypothalamic and pituitary pulses. These have revealed that whilst
10 hypothalamic input is required, its role as a driver of pulsatile pituitary hormone secretion
11 varies between pituitary axes. The organisation of pituitary cells has a key role in modifying
12 their response to hypophysiotropic factors, which can lead to a memory of previous demand
13 and enhanced function. Feedback can lead to oscillatory hormone output that is independent of
14 pulses of hypophysiotropic factors and instead results from the temporal relationship between
15 pituitary output and target organ response. Thus, the mechanisms underlying the generation of
16 pulses can not be generalised and the circularity of feedforward and feedback interactions must
17 be considered to understand both normal physiological function and pathology. We describe
18 some examples of the clinical implications of the recognition of the importance of the pituitary
19 and target organs in pulse generation and suggest avenues for future research in both the short
20 and long-term.

21

22 **Précis**

23 Le Tissier and his colleagues revisit Harris's “Neurohumoral theory” to reassess the
24 contribution of individual components of hypothalamic-pituitary-target organ axes in hormone
25 pulse generation.

1 **Introduction**

2 Understanding the origin of anterior pituitary hormone pulses in health and how they are
3 disturbed in disease is a long-standing question (1). The accepted “textbook” view has been that
4 hypothalamic hormones are the dominant factors generating these pulses, based largely on the
5 seminal experiments of Geoffrey Harris and colleagues that led him to develop the
6 “Neurohormonal Theory” (2). Over sixty years later, the importance of hypothalamic factors is
7 still unquestioned, however, it is apparent that their role in pituitary pulse generation is more
8 complex than previously assumed. It is now clear that no single model system exists and that for
9 each pituitary axis pulses of hormones are generated by a combination of hypothalamic input
10 (3), pituitary response (4), short loop feedback (5) and target organ feedback (6). A clearer
11 understanding of these interactions allows definition of their orchestration, essential for
12 understanding the circuitries underlying physiology and behaviour (7).

13 In this review we will principally consider ultradian pulses of anterior pituitary hormones and
14 divide their generation into two components: the regulatory inputs to the pituitary, both from
15 the hypothalamus and peripheral organs; and the response of the pituitary gland. We will use
16 specific examples to describe the processes and interactions involved and how their
17 modification may lead to pathology. We will focus on the mechanisms underlying the generation
18 of ultradian pulses. This review will not address the circadian pattern of pituitary hormone
19 output: instead the reader is referred to three relevant articles/reviews (8-10).

20

1 **Hypothalamic and target organ input in the generation of pituitary** 2 **hormone pulses.**

3 *Hypophysiotropic neurons share features with many other neuronal cell types*

4 The parvocellular hypothalamic neurons, which store and secrete hypophysiotropic hormones,
5 have largely been considered as a separate class of neuron from those in other regions of the
6 brain that have traditionally been classified by their small neurotransmitters. This view has
7 now changed with studies of neurotransmitter contribution to the regulation of neurohormone
8 release (11,12) and the realisation that many other neuronal circuits can be classified by their
9 secretion of neuropeptides whether they have (e.g. somatostatin (SST) (13)) or do not have (e.g.
10 kisspeptin (14) and orexin (15)) a neuroendocrine role. A unique feature of parvocellular
11 neurons is that they lack post-synaptic targets, however, it has been shown that neuronal (16)
12 and endothelial cell (17) inputs can modulate GnRH nerve terminal activity at the median
13 eminence, similar to retrograde signalling at synaptic terminals. This highlights that the
14 mechanisms and interactions which regulate hypothalamic parvocellular neurons should not be
15 considered in any way distinct from those of other brain regions. A further recent realisation is
16 that neurons considered as single populations may exist as multiple subtypes, which has been
17 shown by single cell transcriptomics (18,19) and this is also true of hypophysiotropic neurons:
18 regionally distinct gonadotrophin releasing hormone (GnRH) neurons have differential roles in
19 GnRH pulse and surge generation (reviewed in (20)); and functional studies have identified two
20 types of tuberoinfundibular dopamine (TIDA) neurons, only one of which is responsible for
21 regulation of prolactin release (21). Further studies are likely to reveal heterogeneity in other
22 hypophysiotropic neuronal populations with distinct roles in both pituitary regulation and
23 modification of other hypothalamic functions.

24

1 ***Pulsatile hypothalamic output is not required for pulsatility in all pituitary axes***

2 It is becoming increasingly clear that the previously accepted concept of the hypothalamus as
3 the source of anterior pituitary hormone pulse generation is not applicable to all axes (Figure
4 1). This concept, of a simple hierarchal pulse generating relationship, is based on seminal
5 studies in multiple species showing a concordance of the pattern of GnRH output and that of
6 pituitary luteinising hormone (LH) and follicle stimulating hormone (FSH) (22-24). Afferent
7 inputs to GnRH neurons are the origin of pulse generation (25), as demonstrated by a series of
8 extensive and elegant studies of the GnRH system (20). As a consequence, the GnRH system has
9 provided a paradigm for pulse generation in other pituitary axes, especially where the pattern
10 of hypothalamic output can not be robustly measured. Identification of the factors regulating
11 other axes show a further level of complexity, with multiple hypothalamic factors having
12 synergistic (eg corticotrophin releasing hormone (CRH) and vasopressin (26)) or antagonistic
13 (eg growth hormone releasing hormone (GHRH) and SST (27)) actions that affect the amplitude
14 or duration of a pituitary hormone pulse but do not contradict the hierarchal relationship.
15 However, recent studies in the adrenal axis has questioned the requirement for pulsatile
16 hypothalamic input for a corresponding pulsatile pituitary output: constant CRH stimulation in
17 conscious, freely moving rats resulted in pulsatile adrenocorticotrophic hormone (ACTH) and
18 corticosterone release with a frequency unaltered from endogenous pulses (28). Similarly, in
19 the thyroid axis constant infusion of thyrotrophin releasing hormone (TRH) in humans has been
20 shown to result in pulses of TSH (29). This is not to say that TRH and CRH are not released into
21 the portal circulation in pulses, where measurement has been made release is pulsatile (30-33)
22 but this may be more related to maintaining responsiveness of target cells rather than pulse
23 generation *per se* (34). Thus the paradigm established by the GnRH-gonadotrophin-sex
24 hormone relationship (Figure 1 , left) may not hold for other axes, such as CRH-ACTH-cortisol
25 (Figure 1, right), or indeed fully account for the relationship of GnRH and gonadotroph output at
26 the time of the LH surge (20). Measurement of other hypophysiotropic factors with sufficient

1 temporal resolution to determine their relationship with pituitary hormone output or
2 optogenetic manipulation of their hypothalamic neurons are required to determine this.

3 ***The electrical activity required for neurohormone release can be defined but is modified***
4 ***with physiological status***

5 In those pituitary axes where pituitary hormones are released in pulses with a frequency of 10s-
6 100s of minutes, it has not (to date) been possible to directly correlate the patterns of
7 hypothalamic neuron electrical activity with their hypophysiotropic secretion. Calcium imaging
8 and optogenetic manipulation to impose electrical activity with concurrent monitoring of
9 pituitary hormone output (assumed to reflect hypothalamic factor release) have been used as
10 alternative approaches to determine the minimal frequency and duration required to drive
11 neurohormone secretion. This has been successfully applied to GnRH neurons, demonstrating
12 that stimulation at 10 Hz (but not at frequencies below 5 Hz) are required for a duration of 2
13 minutes (but not 30 seconds) for generation of LH pulses (35), and to kisspeptin neurons,
14 identifying them as a source of the GnRH neuron pulse generator (25,36). These studies assume
15 that electrical activity and neurohormone release are correlated, which is likely in the short
16 term, however studies of TIDA neuron electrical activity with simultaneous recording of
17 dopamine output has demonstrated that this may not be true with changes in physiological
18 status (37). In lactation, prolactin feedback no longer leads to dopamine release from TIDA
19 neurons, which maintains the high level of the lactogen, but unexpectedly still leads to increased
20 electrical activity. A similar disconnect between electrical activity and neurohormone release
21 may occur in kisspeptin neurons which have an altered optogenetic stimulatory requirement in
22 diestrus and ovariectomized females (36) and a loss of neurohormone expression in lactation
23 (38).

24 ***Hypothalamic neuron coordination is required for pituitary regulation***

25 Whatever the requirement for pulsatile hypothalamic output to generate pituitary hormonal
26 pulses, there is an absolute requirement for hypophysiotropic regulation of the pituitary for

1 normal physiological function. This requires coordinated release from multiple neurons to
2 ensure a sufficient concentration of neurohormone in the portal circulation to elicit a response
3 from pituitary cells; for example, it has been shown that a minimum of 60 GnRH neurons are
4 required for pulsatile LH release in mice (35,39) but five times that number are required for
5 surge generation (40). More direct evidence for coordination has been shown by monitoring
6 pairs of TIDA neurons, where the electrical activity of a proportion of cells are coordinated over
7 a period of minutes (37). In both cases, there is an implication that a subset of the neuronal
8 population is active at any one time, which may be important in avoiding fatigue. This provides
9 a rationale for a large reserve population but also a requirement for interneuron coordination
10 over both space and time; for example, a multi-layered spatial and temporal coordination of
11 TIDA neurons remains stable over a period of days, which may underlie the sustained dopamine
12 release required for inhibition of prolactin secretion (41). Such multi-layered organization of
13 neuronal spiking frequencies are widely used for other brain-body functions, such as sleep, in
14 both mammalian animal models and humans (42).

15 ***Intrinsic and extrinsic mechanisms coordinate hypothalamic neuron activity***

16 The coordination of hypothalamic populations regulating pituitary secretion can occur through
17 a number of mechanisms. In other brain systems, including those of other parvocellular
18 neuronal systems, negative feedback and feedforward loops act as relatively simple networks to
19 coordinate population activities (43) and there is evidence for a similar network-driven
20 coordination of the hypophysiotropic neurons. These can be divided into intrinsic interactions
21 within a population and extrinsic coordination requiring input from other neuronal cell types.
22 Whilst there is evidence for both (as described below), a combination is likely to ensure the
23 coordination required for robust pituitary regulation.

24 ***Intrinsic coordination***

25 Coordination of the electrical activity of hypophysiotropic neurons has been reported, in
26 particular for dopamine (37,44) and cultured GnRH neurons (45). Whilst this may suggest a role

1 for electrical coupling through gap junctions, these have been shown to be absent in both mouse
2 TIDA (37) and GnRH (46) neurons. There may be species differences, however, since in rat TIDA
3 neurons electrical coupling mediated by gap junctions has been described (44). An alternative
4 mechanism underlying intrinsic coordination is chemical coupling and there is evidence for this
5 regulating TIDA neurons via negative feedback loops, with TIDA neurons both releasing and
6 responding to GABA (47). In addition, dopamine 2 receptor (D2R) at the TIDA neuron cell body
7 mediates an ultrashort feedback loop leading to oscillatory activity in rats (48). A similar
8 ultrashort autoregulatory loop has been described for GnRH neurons, which express GnRH
9 receptors and have altered electrical activity in response to GnRH (49).

10 *Extrinsic coordination*

11 Input from the higher brain centres regulating hypophysiotropic neurons will obviously
12 coordinate their activity, however, there is a clear role for intrahypothalamic regulation (50)
13 and it is well recognised that SST and kisspeptin have important regulatory roles in GHRH (51)
14 and GnRH output (14) respectively. Recent studies have determined specific roles for these
15 extrinsic factors and defined key steps in their regulation of neurohormone output. The
16 inhibitory action of SST has been shown to counterintuitively lead to stimulation of GHRH
17 neurons as a result of an initial fast and transient direct inhibition of the GHRH neuron itself,
18 followed by a delayed inhibition of both excitatory glutamate and inhibitory GABA inputs (52).
19 Optogenetic manipulation has identified kisspeptin as the GnRH pulse generator (25) and other
20 studies have shown that firing of kisspeptin neurons is modulated by steroid feedback (53).
21 Thus, feedforward loops are key features of both of these hypophysiotropic systems. A further
22 complexity in the extrinsic inputs regulating hypophysiotropic output may be their subcellular
23 location. Kisspeptin has been shown to have differential effects at the GnRH cell body compared
24 with the nerve terminals at the median eminence (16), where a role for local endothelial nitric
25 oxide production has been suggested as a local synchronising signal (54).

26 ***The median eminence plays a role in coordinating and modifying hypothalamic output***

1 The final step in the output of hypophysiotropic hormones is their release at the median
2 eminence (ME). Release from a large number of neurons into this richly vascularised structure,
3 with convoluted loops collecting output from a large release area, optimises both the amplitude
4 and duration of neurohormone pulses in the portal circulation, whilst avoiding neuronal fatigue
5 and exhaustion (41). In addition to roles in the coordination of hypophysiotropic factor release
6 (see above), the ME may actively modify output by alteration of access of nerve terminals to the
7 rich capillary bed by either changes in localisation, which has been shown to vary with age for
8 GHRH neurons (55), or tanyocyte ensheathment, shown for GnRH neurons to vary at different
9 stages of the oestrous cycle (56).

10 ***Peripheral inputs can generate pituitary hormone pulses***

11 The importance of target organ feedback in the regulation of hypothalamic-pituitary axes is well
12 recognised and incontrovertible, balancing the feedforward regulation by hypothalamic and
13 pituitary factors. An excellent example of this is the differential regulation of LH and FSH by
14 ovarian inputs, with reduced inhibin and increased progesterone feedback actions on the
15 pituitary generating a second phase of FSH (but not LH) at proestrus and estrus (reviewed in
16 (22)) (Figure. 1, left panel).

17 Remarkably, recent studies inspired by mathematical modelling have shown that target organ
18 feedback itself can act as a pituitary hormone pulse generator, as the fast feedforward action of
19 ACTH on the adrenal gland and delayed feedback of glucocorticoids can generate pulses of both
20 hormones with invariant CRH (reviewed in(6)) (Figure 1, right panel). Since an intra-adrenal
21 glucocorticoid feedback loop has recently been suggested from modelling (57), this suggests
22 that the adrenal gland itself may be the primary pulse generator in the hypothalamic-pituitary-
23 adrenal axis in the absence of stress. The extent to which similar temporal relationships of
24 hypothalamic-pituitary regulation and feedback exist for other axes is currently unclear,
25 although a delayed feedback of prolactin on dopamine neurons (41) may have an important

1 impact on dopamine tone, facilitate increased secretion of prolactin and lead to the reported
2 ultradian pulses of basal prolactin secretion (58).

3 The potential interactions whereby feedback can generate or modulate pulsatile pituitary
4 hormone secretion are complex and may include:

- 5 • the temporal relationship between the feedforward and feedback regulation, which is
6 complicated by the feedback occurring at multiple levels; for example, the differential
7 feedback actions of ovarian steroids are mediated by rapid non-genomic and classical
8 steroid receptor actions in both the hypothalamus and pituitary during the oestrous
9 cycle, with effects that are dependent on receptor isoform expression and downstream
10 signalling (reviewed in (22,24)).
- 11 • the sensitivity of the system to feedback, exemplified in the thyroid axis, where
12 differential expression of thyroid hormone receptor beta isoforms results in its relative
13 increase in sensitivity to thyroid hormones (59), providing an anticipatory mechanism
14 to protect peripheral organs from overexposure to these hormones (34).
- 15 • differential feedback at the level of the hypothalamus and pituitary. Again the thyroid
16 axis provides an excellent example of this, since feedback to the hypothalamus is
17 dependent on active transport of thyroid hormone at the level of the median eminence
18 (60) but is enhanced by post-translational modification of type 2 deiodinase (61).
- 19 • multiple factors feeding back on a single cell type; for example, dopamine neurons will
20 be exposed to GABA, dopamine and prolactin feedback, with different time scales
21 (41,47,48).

22 Thus, it is possible that feedback occurs at both pituitary and hypothalamic levels and at
23 multiple sites within each organ, through the action of multiple factors on a single cell type, or
24 both. This is further complicated when consideration of feedback to higher brain centres is
25 included; for example, glucocorticoid feedback on the limbic system and brain stem (62).

26

1 **Intrapituitary regulation of hormone pulse generation**

2 It is perfectly feasible that the anterior pituitary gland would simply passively respond to
3 hypothalamic and peripheral inputs, with its cells simply acting as an amplifier of hypothalamic
4 regulation that is modulated by feedback from target organs. However, it is now apparent that
5 this is not the case, with an active role for the pituitary mediated by the structural organisation
6 of its component cell types affecting how they receive, interpret and translate hypothalamic and
7 peripheral signals into highly ordered hormone pulses. This was previously suggested by a
8 disconnect in the output of dispersed pituitary cells compared with those in the intact gland
9 (63) but is being increasingly demonstrated and dissected using a combination of mouse models
10 and technological innovation which allows both temporal and structural imaging (64,65).

11 *Pituitary cells are organised as intermingled homotypic cell networks.*

12 Large-scale 3D imaging of genetically-modified mouse models expressing fluorescent proteins
13 under hormone promoter control has revealed the structural and functional organisation of the
14 pituitary gland and its rich vascularisation (4). This has described the developmental program
15 of the topological organisation of differentiated cells throughout the gland (63,66) from early
16 fetal life to adulthood, and has demonstrated a role for early-differentiated cells (e.g.
17 corticotrophs) in controlling both the positioning and expression of late-differentiated cells (e.g.
18 gonadotrophs) (67). Contact between homotypic cells and organisation of characteristic
19 morphological features occurs soon after endocrine cell differentiation, before the onset of
20 hormone secretion and leads to cell network formation (67,68). Among the signal molecules
21 involved in cell network architecture and plasticity, detailed analysis of the cadherin family has
22 revealed a 'bar-coding' expression of cadherins within distinct pituitary cell populations from
23 both mouse models (69) and humans (70), which has also been proposed as a marker rule for
24 discriminating invasiveness in GH and PRL adenomas.

25 Pituitary cell networks share fundamental properties with other biological networks including
26 metabolic signalling networks in yeast and bacteria (71), where a prevalent feature is that of

1 simple assembles of elements (so-called network motifs) which recur within the population
2 (72). The organisation of the various pituitary cell types into distinct motifs suggests that there
3 will be differences and similarities in their role in axes function. The spatial organisation, and
4 its plasticity throughout life, is exemplified by the GH and prolactin cell networks. Upon sexual
5 maturation there is a transient increase in the generation of multiple clusters of contacting GH
6 cells (illustrated in Figure 2) in males but not females in the wings of the pituitary, coincident
7 with an increase in the highly ordered GH pulses which control liver insulin-like growth factor 1
8 production (64,68). The importance of these GH cell clusters as network motifs which lead to
9 increased body growth is suggested by the correlation of their formation with growth rate (68)
10 and the finding that GH-deficient animals are normal in size if the GH cell clusters are preserved
11 (73). In contrast, PRL cells are organised as multiple honeycomb network motifs (like an orange
12 peel) which are more prominent in lactating females and display experience-dependant
13 plasticity as they remain after weaning (74). This altered network organisation has been shown
14 to result in enhanced prolactin output in subsequent lactations (74) and may have a role in
15 reducing the tonic output of prolactin in reproductively experienced rats (75) through an
16 enhanced response to dopamine inhibition (76).

17 *The vasculature has a role in signal input to pituitary cell networks*

18 Networks of endocrine cells do not work alone but form a functional continuum with other
19 elements within the pituitary gland, including the vasculature. Network motifs and the rich
20 plexus of fenestrated capillaries are topologically organized in a manner which is distinct for
21 each endocrine cell type (Figure 2), and may therefore reflect their different secretory temporal
22 dynamics (63). Initial cell network formation begins before the first capillaries invade
23 embryonic pituitary tissue (67) and loss of the pituitary cell transcription factor Prop1 leads to
24 a failure of organ vascularization (77). Thus, endocrine cell networks have a stimulatory and
25 organisational role in patterning capillary invasion.

1 The organisation of the vasculature with pituitary endocrine networks may have a significant
2 impact on the amplitude and timing of exposure of pituitary cells to hypothalamic regulatory
3 factors since seminal studies of the portal vasculature have shown that hypophysiotropic nerve
4 terminals specifically abut portal vessels which irrigate specific pituitary regions (78,79). In
5 addition, there is a highly dynamic regulation of the distribution of incoming secretagogues
6 within the pituitary through altered blood flow dynamics within the capillary bed of the
7 pituitary (80) but rapid transit of signalling molecules (in a range of seconds) throughout portal
8 fenestrated capillaries (81). This suggests differential timing of exposure of different regions of
9 the pituitary to hypophysiotropic factors, resulting in a complex dynamic of sequential
10 stimulation, with “scout cells” stimulated before other cells within a homotypic network. Since
11 networks have a functionally coordinating role (see below), this pattern of exposure may lead to
12 synergistic interactions and potential role(s) for specific subsets of cells ensuring robust
13 responses to stimulation. The contribution of the blood system to pituitary hormone pulsatility
14 also involves the fate of hormones from their releasing site towards the bloodstream, which will
15 ultimately deliver the appropriate pattern of hormone pulses to the peripheral target, as well as
16 coordination of oxygen and nutrient supply with the metabolically demanding processes of (80).

17 *Pituitary networks coordinate response to regulation*

18 A functional role for homotypic pituitary cell networks in determining endocrine output is
19 suggested by their formation before the onset of hormone secretion and stimulation by
20 secretagogues and functional reorganization in response to altered demand (68,74). This has
21 been confirmed by *ex vivo* analysis of calcium and gene expression dynamics in homotypic cell
22 networks, with coordinated responses to stimulation that are severely dysregulated when
23 networks are disrupted (64,73,82,83). Gap junction coupling contributes to this network
24 coordination (64,74,82), however, this does not preclude roles for paracrine factors between
25 both homotypic and heterotypic cells (reviewed extensively in (84)), such as secreted TSH
26 which exerts an ultrashort negative feedback which could drive ultradian TSH pulses (34). It is

1 also possible that pituitary networks mediate predictive programming, or priming, of axis
2 function since in prolactin cells, the increased organisation associated with lactation persists
3 for months after weaning and leads to enhanced function (74). Similarly, the increased
4 clustering of somatotrophs at puberty could be considered as a priming event for increased GH
5 release, although in this case the effect is transient (64). It is possible that similar transient
6 changes in organisation enhance the altered sensitivity and self-priming of gonadotrophs to
7 GnRH stimulation (85) since increased cell movement and number of cell processes have been
8 described in this cell type in response to GnRH (86) and estradiol (87). Thus both structural and
9 functional organisation of pituitary endocrine cells as intermingled 3D cell networks have
10 important roles in the amplitude and dynamics of hormone secretion which can be modified
11 throughout life.

12 *Pituitary cells are heterogeneous*

13 Whilst pituitary networks mediate a coordination of cell activity, individual cells also show
14 functional heterogeneity, which may reflect transient or permanent differences in cell activity.
15 This is exemplified by the identification of a small subset of prolactin cells which act as pace-
16 makers, or network nodes, synchronizing the activity of nearby homotypic network cells (65). It
17 is these pace-making cells which mediate the altered function of prolactin cells between first
18 and second lactation, showing an ability to store a cellular memory of previous demand that
19 also leads to an enhanced output when rechallenged (akin to learning). Over a timescale which
20 is an order of magnitude longer than that of secretory activity, prolactin gene expression in
21 lactotrophs has also been shown to be heterogeneous (82). A continuous distribution of both
22 transcription rates and switches were found in this study, although interestingly this was locally
23 spatially coordinated by the prolactin cell network, suggesting that the mechanisms underlying
24 homotypic cell coordination can act over a wide range of timescales. Similar functional
25 heterogeneity has been described for other pituitary cell types, which can result in stereotypic
26 variable responses to stimulation which have previously been considered to be stochastic (88).

1 Further studies are required to determine if these heterogeneous responses identify a distinct
2 sub-population of pituitary cells or transient activity states, which will likely be identified by
3 high throughput sequencing technologies and/or photolabeling of individual cells in situ (89).

4 *Pituitary cell secretion is integrated to shape pulsatile circulating hormone*

5 The rate of entry of a secreted pulse of pituitary hormone to the bloodstream and exit from the
6 gland will be determined by the relationship of cell networks with the pituitary
7 microvasculature, where perivascular spaces act as gate-keepers for hormone transfer to the
8 capillary lumen (80). Once in the systemic circulation, a pulse of hormone will be combined with
9 that released in previous secretory events, resulting in the concentration of circulating hormone
10 is an integration of basal and pulsatile release which is dependent on hormone half-life (1,4).
11 Since the half-life of pituitary hormones can be modified by circulating binding proteins (90)
12 and post-translational modification (34), both of which also modify their bioavailability, the
13 pattern of exposure of a receptor to a hormone pulse is complex and will not simply mirror that
14 of pituitary release.

15

16 **Implications for health and disease**

17 A recognition that pulses of pituitary hormone are generated and modified at multiple levels has
18 important implications for the study of normal axes function but, importantly, also for how
19 dysregulation occurs and for identification of therapeutic targets. This is particularly relevant to
20 pituitary tumours, where hormone output is largely independent of hypothalamic stimulation.

21 In Cushing's disease, for example, there is a marked increase in basal secretion of both ACTH
22 and cortisol and pulsatility is preserved (91) but becomes less ordered (92). Significantly when
23 considering the interaction between glands, there is a decrease in the potency of cortisol
24 stimulation by ACTH (93) and a reduction in the pulse correlation of the two hormones (91).

25 Similar changes in the orderliness of pituitary hormones has been described for other types of

1 pituitary adenoma, which importantly is largely normalised by surgical but not medical
2 treatment (94).

3 It is possible that the interactions between the hypothalamus, pituitary and target organs in
4 generating pulses may have a significant role in a number of endocrine disorders, and thus
5 should be considered as a potential mechanism leading to disease as well as new targets for
6 therapy. For example, multiple studies to identify defects leading to polycystic ovarian
7 syndrome (PCOS) have focused on dysregulation at the levels of the hypothalamus (95,96) and
8 ovary (97). Aspects of the disorder, such as the potential role of hyperinsulinemia in loss of
9 fertility have been studied at the level of the pituitary (eg (98)) but overall there has been a
10 paucity of studies of the role of the pituitary. It is possible that increased LH pulse amplitude
11 (but not pulse frequency) found in PCOS patients may be a result of an alteration of pituitary
12 function and further research into a potential role of the pituitary in this disorder is warranted.
13 Given that PCOS is a heterogeneous syndrome, it is possible that there are multiple aetiologies
14 that involve all levels of the HPG axis some of which may be secondary to the primary defect
15 but nevertheless require improved understanding and may be targets for therapy.

16 The multi-level regulation of pulsatile hormone output also has important implications for
17 diagnosis of dysfunction. An excellent example of this is provided by the thyroid axis, where the
18 “normal” concentration of circulating TSH can vary between individuals (34) and may be
19 altered for prolonged periods following normalisation of axis function following hyper- or
20 hypothyroidism, referred to as hysteresis (99). This may occur as a result of differential rates of
21 feedback regulation at different levels of the axis; for example, a reduction in hypothalamic TRH
22 gene expression in response to normalisation of hypothyroidism (100) would be expected to be
23 rapid in comparison with any change in pituitary thyrotroph cell mass (101), resulting in an
24 alteration of the set point for each level of the axis.

25

26 **Future research/perspective**

1 ***Models of pituitary pulse generation:***

2 It is clear that there has recently been substantial progress in understanding the contribution of
3 individual components of hypothalamic-pituitary-target organ axes to pulse generation. However, the
4 challenge is to dissect the temporal interactions of these individual components, which requires *in*
5 *vivo* studies with simultaneous monitoring of system inputs and/or outputs. The use of imaging
6 technology to observe cell activity has been instrumental in much of the recent progress, since it
7 allows both temporal and spatial resolution of activities. Further development of *in vivo* imaging
8 technology, such as the use of gradient index lenses (102), is required to facilitate this. An important
9 consideration in these studies is the silencing of multiple hypothalamic neuron populations by
10 anaesthesia (80), meaning that imaging in awake, freely moving animals is required. Furthermore,
11 monitoring of cell stimulation and activity currently relies on imaging of specific cell signals, such as
12 calcium as a surrogate for monitoring both inputs and outputs (eg (64)). The development of
13 methodologies to specifically monitor receptor activation, such as “sniffer cells” (103) or luciferase
14 monitoring of G-protein coupled (104) and cytokine receptor (105) activation may allow more direct
15 measurement of both stimulatory inputs and hormonal output.

16 The heterogeneity of both hypothalamic and pituitary cell populations in generating hormone pulses
17 has been a notable feature throughout this review. Whilst it is possible that this may represent
18 stochastic cell activity in some cases, in others it has been found to be deterministic (88). Since
19 multiple studies have demonstrated that only a small proportion of hypothalamic (eg GnRH (40)) or
20 pituitary (eg GH (73)) cells are required for apparent normal function, the question remains whether
21 the heterogeneous responses reflect sub-populations with specific physiological functions. Identifying
22 the differences in protein expression and post-translational modification that may underlie
23 heterogeneity may be suggested by single cell transcriptomics of cells with specific activities. These
24 studies will not define whether the heterogeneity reflects transient activity or specific sub-population
25 of cells, however they will suggest factors that define sub-populations of cells currently primarily
26 defined by the hormone they produce. The use of cell tracing methodologies, optogenetics and
27 Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which have already

1 made dramatic contributions to understanding of hypothalamic-pituitary axis function (eg (25,106)),
2 will allow confirmation of sub-population identity and study of their function. This will be facilitated
3 by the use of CRISPR/Cas technology in combination with adeno-associated viral delivery of factors
4 to manipulate cell function (107). Such single cell transcriptomic approaches have been successfully
5 applied in other systems, with consequences for identifying novel therapeutic targets (108) and for
6 sub-population identification in the brain (109).

7 Our identification of features akin to memory and learning in the pituitary suggests a potential role of
8 the gland in physiological programming. For example, a persistent alteration in corticotroph activity
9 has been described in adult sheep exposed to a brief period of maternal perinatal undernutrition (110).
10 There is a clear requirement for further investigation of epigenetic alteration of gene expression in
11 such models, however, persistent changes in pituitary cell organisation leading to altered network
12 functions are also possible. This will require *in vivo* analysis, as well as a clearer understanding of the
13 mechanisms underlying network-mediated regulation of axis function. The single cell transcriptomics
14 and cell manipulations described above may allow identification of potential mechanisms underlying
15 network function. Mathematical modelling and tissue engineering may aid understanding of how
16 different network motifs affect cell-cell coordination.

17 Finally, the role of the vasculature in modifying temporal and spatial regulation of pituitary function
18 and clearance of secreted hormone from the gland is an area that requires further analysis which
19 should be possible with optogenetic manipulation or the use of DREADDS. These may also be used
20 to determine how the relationship of pituitary cell networks and the vasculature is altered in adenoma
21 formation (111), as well as in other axes dysfunctions such as PCOS.

22 ***Translation to the clinic:***

23 The mechanisms leading to pituitary hormone pulse generation that are currently being elucidated in
24 rodent models are likely to generally translate to those in humans, however, there are clear species
25 differences in the physiology of pituitary axes (eg prolactin (112)). Analysis of post-mortem pituitary
26 tissue will allow comparison of network organisation and their relationship with the vasculature, as

1 well as the expression patterns of factors identified as intermediates in network function. It is also
2 possible that fresh post-mortem tissue and adenomas from patients will allow some functional
3 analysis of human pituitary function and correlation with that of rodents. The analysis of organoids of
4 pituitary tissue differentiated from induced pluripotent stem cells (113) will most likely establish
5 whether mechanisms underlying rodent network function are recapitulated in humans, as well as the
6 consequences of mutations identified in patients presenting at clinic with pituitary dysfunction.

7 Many of the current protocols for diagnosis of pituitary dysfunction may not fully interrogate the
8 complex interactions leading to pulse generation, which may explain why, for example, current
9 provocation tests misdiagnose GH axis function in a proportion of patients (114). Rodent models will
10 allow the development of tests which can more fully define hypothalamic and pituitary functionality
11 and determination of parameters that are affected when physiology is altered; for example, at puberty
12 and in obesity. It is also possible that an *in vivo* assessment of pituitary function may be possible
13 through an improved understanding of how pituitary blood flow relates to function, as this may be
14 assessed in patients through, for example, ultra-fast ultrasound imaging (115).

15 Identification of pituitary cell networks may also affect whether and how stem cell therapy could be
16 used for treatment of hypopituitarism, which would be simplified in humans through transphenoidal
17 access to the pituitary. Whilst there has been substantial progress in identifying pituitary stem cells
18 (116,117) and developing protocols for differentiation of embryonic and pluripotent stem cells to
19 pituitary tissue (118,119), it is currently unclear whether stem cells would be capable of self-
20 organisation or integration into existing pituitary cell networks. This is further complicated by the
21 identification of functional heterogeneity and programming of cell function by previous demand. A
22 naïve stem cell may be capable of differentiation to a lactotroph and integration into an existing
23 network, for example, but may not be functionally equivalent to a cell exposed to the demands of
24 lactation. Furthermore, the prevalence of pituitary adenomas with aberrant network function reflected
25 in disorganised pulsatile output (94) suggests that a failure to fully integrate or recapitulate normal
26 network function may be a risk for the development of pathology. Injection of lineage traced stem

1 cells into the pituitaries of rodent models of hypopituitarism and functional imaging of their function
2 may establish the potential for stem cell therapy in humans.

3 **Conclusion**

4 The elegant and ground-breaking experiments of Harris and colleagues were prescient in their
5 use of *in vivo* models which allow multi-organ interactions. It is now clear that in such an
6 interactive system the concept of a hierarchy is not appropriate except in the identification of a
7 pulse generator, which in the case of the HPA axis, at least, may not be the hypothalamus. This
8 does not suggest that the mechanisms and principles underlying the relationship of the
9 hypothalamus, pituitary and target organs differ between axes but the strength and timing of
10 inputs lead to unique features. Thus, the concepts underlying Harris's Neurohormone Theory of
11 regulation of pituitary axes have borne the test of time but new levels of complexity have
12 emerged that require consideration of interactions between multiple components of the axes.

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19

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1 **Figure legends:**

2 **Figure 1. A simplified schematic showing the contrasting regulation of pituitary hormone**
3 **pulse generation between the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-**
4 **pituitary-adrenal (HPA) axes.** In the HPG axis, the pulse generator is localised in the
5 hypothalamus, where afferent inputs from kisspeptin neurons (25) and neuronal feedforward
6 loops (53) lead to pulsatile release of gonadotrophin releasing hormone (GnRH). This results on
7 the release of pulses of luteinizing (LH) and follicle stimulating (FSH) hormone, stimulating
8 secretion of steroids from the gonads which feedback on a relatively slow timescale to both the
9 pituitary and hypothalamus. In contrast, in the HPA axis the rapid actions of
10 adrenocorticotrophic hormone on the adrenal gland and delayed feedback of glucocorticoids on
11 the anterior pituitary is the source of pulse generation, with corticotrophin releasing hormone
12 (CRH) having a modulatory role (28).

13

14 **Figure 2. The growth hormone (GH) cell network and its relationship with the**
15 **vasculature.** Two-photon imaging of the pituitary of a GH-GFP transgenic mouse with
16 capillaries labelled with gelatine-rhodamine (red). GH cells are organised into a homotypic
17 topologically organised network of cell clusters which are linked by strings of single cells. The
18 cell network is closely associated with capillaries, which are aligned with strings of cells and
19 surround the clusters.



