60 YEARS OF NEUROENDOCRINOLOGY

The structure of the neuroendocrine hypothalamus: the neuroanatomical legacy of Geoffrey Harris

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Abstract

In November 1955, Geoffrey Harris published a paper based on the Christian A Herter Lecture he had given earlier that year at Johns Hopkins University in Baltimore, MD, USA. The paper reviewed the contemporary research that was starting to explain how the hypothalamus controlled the pituitary gland. In the process of doing so, Harris introduced a set of properties that helped define the neuroendocrine hypothalamus. They included: i) three criteria that putative releasing factors for adenohypophysial hormones would have to fulfill; ii) an analogy between the representation of body parts in the sensory and motor cortices and the spatial localization of neuroendocrine function in the hypothalamus; and iii) the idea that neuroendocrine neurons are motor neurons and the pituitary stalk functions as a Sherringtonian final common pathway through which the impact of sensory and emotional events on neuroendocrine neurons must pass in order to control pituitary hormone release. Were these properties a sign that the major neuroscientific discoveries that were being made in the early 1950s were beginning to influence neuroendocrinology? This Thematic Review discusses two main points: the context and significance of Harris's Herter Lecture for how our understanding of neuroendocrine anatomy (particularly as it relates to the control of the adenohypophysis) has developed since 1955; and, within this framework, how novel and powerful techniques are currently taking our understanding of the structure of the neuroendocrine hypothalamus to new levels.

Introduction

Geoffrey Harris the anatomist

Anatomy played a major role throughout Geoffrey Harris's career. When he was a graduate student with Henry Harris, the chair of the Department of Anatomy at Cambridge, he was awarded the Marmaduke Shield Studentship in Anatomy. He then held positions in Cambridge University's Department of Anatomy and then the Physiological Laboratory before moving in 1952 to the newly created Laboratory of Neuroendocrinology at the Maudsley Hospital in South London. Geoffrey Harris's final move in 1962 was to the University of Oxford when he became Dr Lee's Professor of Anatomy; there

he established and directed the MRC Neuroendocrinology Unit. Harris was also a strong advocate and enabler of improving the methods that were used for teaching anatomy at Oxford (Vogt 1972).

A hallmark of Harris's research was a continuous emphasis on establishing structure–function relationships between the hypothalamus and the pituitary gland. This focus played a major part in his ability to consolidate his neurohumoral control hypothesis for the adenohypophysis. Harris recognized early on that establishing how the hypothalamus and pituitary gland were functionally connected required strong interactions between physiological and anatomical experiments. Like the approaches of other pioneering neuroendocrinologists in the UK and USA, Harris's approach contrasted sharply with that used by 'neurosecretionists' (Ernst and Bertha Scharrer, Howard Bern, Manfred Gabe, etc.). These workers attempted to use morphology alone to establish neurosecretion as a process that was quite distinct from the other forms of chemical signaling that are used by neurons in the brain (Watts 2011).

While he was in the Cambridge University Physiological Laboratory in the late 1940s, Harris focused much of his work with John Green on clarifying the structural arrangement of the hypophysial portal vasculature (Green & Harris 1947, 1949). His reason for doing so was that it was impossible to establish that humorally mediated control was a viable explanation without first determining the structural means though which this control was exerted. What followed during the next 5 years was a series of very elegant and technically demanding experiments that began to establish the primacy of the hypophysial portal vasculature for conveying chemical signals from the hypothalamus to the adenohypophysis (Harris 1950, Harris & Jacobsohn 1950, 1952). Harris's interpretations were certainly not universally accepted in 1955, but they were greatly strengthened by Nikitovich-Winer & Everett (1957) later in the decade.

Neural control of the pituitary gland

All of this work was discussed at various scientific meetings, which were documented in three substantial reviews (Harris 1948, 1951a,b), before it was presented in a more extensive form in *Neural Control of the Pituitary Gland* (Harris 1955a). For decades, *Neural Control of the Pituitary Gland* has been recognized as a landmark publication in the development of neuroendocrinology. The book was published as the third contribution to what would

become the long-running Monographs of the Physiological Society series.

Harris stated that the book was an:

'attempt to analyse the mechanism by which the central nervous system, and the hypothalamus in particular, controls and integrates the activity of the [pituitary gland]'.

Harris (1955a, p 5)

As such, it offered a comprehensive review of the contemporary state of the anatomical and functional bases of neuroendocrinology. But as far as the adenohypophysis was concerned, Harris's neurohumoral control hypothesis was still far from being universally accepted when the book was published (cf. Zuckerman 1956, Sayers et al. 1958). Indeed, this period was one of very vocal debate on the topic, with strong advocates in each of the opposing camps. Most famously Sir Solly Zuckerman dismissed the book in his review in *Nature* as 'an edifice of speculation' (Zuckerman 1956). (Zuckerman's review is well worth reading in its entirety to get a sense of the debate that was going on at this time.) The book was therefore not simply a description of a well-established concept. Instead, it was a carefully presented account of the contemporary research results that Harris and others were producing in order to understand how the brain controls the pituitary gland.

Neuroendocrinology as neuroscience

From the standpoint of exactly how the hypothalamus controls the adenohypophysis, Harris was not particularly explicit in the book about the detailed mechanisms:

The information as to the details of the mechanism involved is scanty but it seems likely that nerve fibers in the hypothalamus liberate some humoural substance into the primary plexus of the vessels, and that this substance is carried by the vessels to affect anterior pituitary activity.

Harris (1955a)

Experiments were only just beginning to examine the nature of the chemical signals, which parts of the hypothalamus were responsible for controlling which pituitary hormones, how they were controlled by inputs to the hypothalamus and the rest of the brain, and so on. Instead, the book presents a systematic account of the anatomical arrangement of the connections between the hypothalamus and the pituitary gland together with

evidence that the functional connection to the adeno-hypophysis must be neurohumoral.

Neural Control of the Pituitary Gland was published at a time when many of the seminal discoveries that would eventually shape neuroscience as we now know it were being made, including: Eccles's confirmation of the primacy of chemical neurotransmission at spinal moto-neuron synapses (Brock *et al*. 1952); the formal description of the ionic basis for action potential propagation (Hodgkin & Huxley 1952) and synaptic transmission (Fatt & Katz 1952); the first visualization of the synapse using electron microscopy (Palade & Palay 1954); and the introduction of the Nauta stain for tracing neural pathways (Nauta & Gygax 1954). The book was therefore published when the physiological basis of chemical neurotransmission was on the minds of many people and was already starting to influence neuroendocrinologists.

Harris presented his view about the hypothalamic control of the pituitary gland from what we would now recognize as a neuroscience perspective rather more clearly in a review entitled 'The function of the pituitary stalk' (Harris 1955*b*), which was published in November of 1955. That paper was developed from the Herter Lecture that Harris delivered at Johns Hopkins University in March 1955. It was most likely written later than *Neural Control of the Pituitary Gland*, the preface of which says that the book was based on a series of teaching lectures that Harris gave in Cambridge before he moved to the Maudsley in 1952.

Harris's thoughts about hypothalamo–hypophysial interactions are better developed in the Johns Hopkins paper, and he makes clearer statements about the topic there than he did in his book. The way in which he discusses these control processes shows that he was fully aware of the rapidly developing progress that was being made in chemical neurotransmission, pathway organiz-ation, functional localization, and other concepts that we now associate with neuroscience. It seems to have been the first time that he presented a view of hypothalamic control of the pituitary in this manner.

Harris discussed three concepts or properties that he considered essential for understanding how the hypothala-mus controls the pituitary gland (Harris 1955*b*), including:

i) three criteria that all releasing factors (hormones) must fulfill if they are to be regarded as mediators of chemical signal transmission to the adenohypophysis;

ii) the spatial localization of function in the hypothala-mus; and

iii) the pituitary stalk as a final common motor pathway to the pituitary gland.

He continued to investigate and refine these properties for the rest of his career (cf. Harris 1972), and they have had a major influence on neuroendocrinology ever since. In the next section, we discuss in more detail how each of these three concepts has influenced the progress in understanding neuroendocrine anatomy since Harris first presented them in 1955.

The structure of the neuroendocrine hypothalamus

Chemical neurotransmission and the releasing factors

The nature of neurotransmission at central, autonomic, and neuromuscular synapses, together with the chemical nature of the signals that mediate these processes had to have been on Harris's mind in 1955. The view that the most well-accepted neurotransmitters at the time – acetylcholine and the adrenergic catecholamines – con-tributed to the hypothalamic control of gonadotropin secretion had already been presented by Charles Sawyer and his colleagues in the USA a few years earlier (Sawyer *et al*. 1949, Markee *et al*. 1952; see also Watts (2011)). Wilhelm Feldberg had made seminal contributions to establishing acetylcholine as a neurotransmitter (e.g., Feldberg 1951), and he was a colleague and collaborator of Harris's (Feldberg & Harris 1953). Presumably, there must have been opportunities for the two of them to discuss chemical neurotransmission and the emerging concepts of neuroendocrinology.

In 1955, Harris stated three requirements that must be met for a compound to be accepted as a releasing factor of adenohypophysial hormones:

'Several suggestions have been put forward as to the nature of a transmitter substance, but such suggestions and the neurohumoral view as a whole will only be established if it is possible to (…)

a) show this substance is present in the blood in the hypophysial portal vessels in greater amount than in systemic blood,

b) show that the concentrations of this substance in the blood of the hypophysial portal vessels varies according to electrical or reflex activation of the hypothalamic nerve tracts,

c) demonstrate that activity of the adenohypophysis is correlated with this varying concentration'.

Harris (1955*b*, p 368)

Although they provided a framework for much of Harris's later work, it was not until 20 years later (and 5 years after his death) that all three requirements were fulfilled for a releasing hormone: GnRH (Fink 1976, Sarkar *et al.* 1976). More generally, they are logical criteria for any chemical signal, and in this context, they make an interesting comparison to those criteria first proposed for neurotransmitters by Paton (1958). Paton was the Chair of the Pharmacology Department at Oxford University at the same time that Harris was the Chair of the Human Anatomy Department.

Harris mentioned 'adrenergic substance', histamine, and other compounds, including 'neurosecretory material associated with the neurohypophysis', as potential neurochemical signals that could control the pituitary (Harris 1955*b*). However, he was careful to say that evidence was not yet available to make any firm statements about the chemical nature of the signals in the hypophysial vasculature (Harris 1955*a*,*b*). It took another 15 years before the structure of the first adenohypophysial-releasing factors was determined and then a further 5 years before these findings had a significant impact on experiments that could finally elucidate the fine structure of the neuroendocrine hypothalamus.
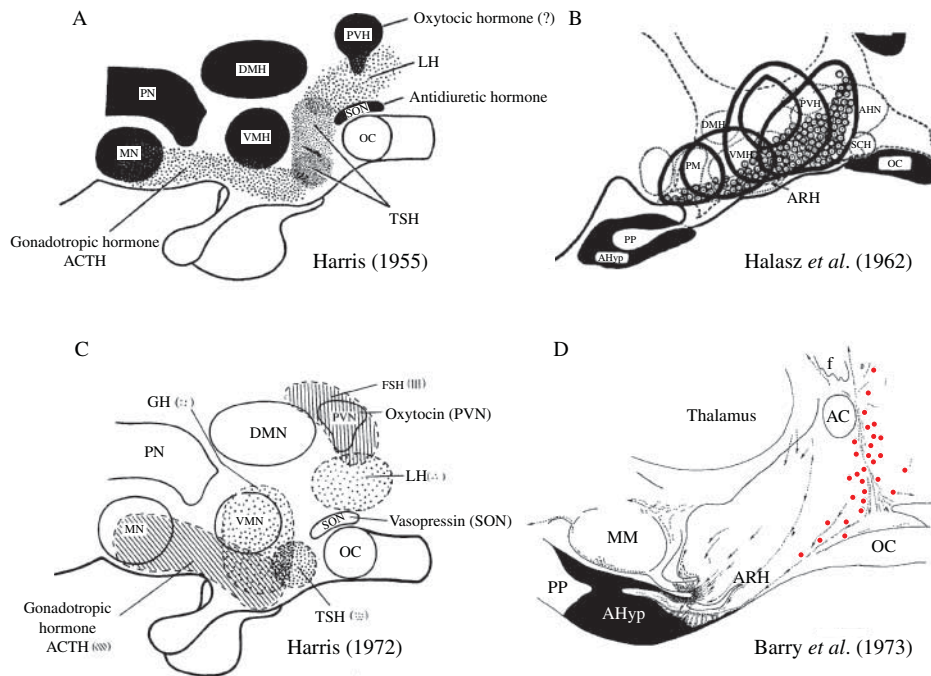
As morphological techniques became more sensitive and sophisticated, it became possible to examine the organization of hypothalamic neuroendocrine neurons in detail (see section Spatial localization of function in the neuroendocrine hypothalamus). Once this happened, an unexpected finding was observed: all of these neurons seemed to express other chemical signals in addition to the primary signal that was active in the pituitary gland. These included other peptides (Sawchenko *et al.* 1984, Everitt *et al.* 1986, Hökfelt *et al.* 1986, Coen *et al.* 1990). More recent evidence has supported the idea that some neuroendocrine neurons also express fast-acting single amino acid-derived neurotransmitters (Hrabovszky *et al.* 2005, Krashes *et al.* 2014).

The presence of these multiple chemical signals in neuroendocrine neurons quickly led to the idea that differential regulation by hormone feedback and various stimuli provided a way for their release mechanisms to switch between different chemical signals and to thereby increase their response adaptation (Swanson 1983, 1991). Differential switching of peptide biosynthetic mechanisms was then demonstrated in CRH neuroendocrine neurons in the hypothalamic paraventricular nucleus (PVH) in response to combinations of glucocorticoid and various stressors (Sawchenko & Swanson 1985, Watts & Sanchez-Watts 1995, 2002, Watts 2005).

Spatial localization of function in the neuroendocrine hypothalamus

Experimental support for the spatial representation of function in the brain emerged during the late nineteenth century with the first reports about the localization of cortical function (e.g., Ferrier 1876, 1890). In his 1955 Johns Hopkins review, Harris made an explicit comparison between the representation of the various parts of the human body in the sensory and motor cortices, on the one hand, and the location of control mechanisms within the hypothalamus for the different pituitary hormones, on the other hand. He then went on to summarize contemporary knowledge – poor as it was – about the hypothalamic locations of these control functions by presenting the first map to show which parts of the hypothalamus controlled the various pituitary hormones (Fig. 1A; Harris 1955*b*). The map was based on results from what were then the only experimental techniques available for investigating the locations of brain functions: lesions and electrical stimulation. It was followed 7 years later by a map published by Halasz *et al.* (1962) that was derived from pituitary transplantations (Fig. 1B). In 1971, Harris presented a second version of his map (Fig. 1C; Harris 1972). Although he modified some of the locations associated with particular pituitary hormones, Harris changed little in the second map from the first one, despite a 16-year interval and the transplantation studies from the Hungarian group (Halasz *et al.* 1962, Szentágothai *et al.* 1968). What was the reason for this?

Identifying with any detail the hypothalamic origins of the chemical signals that are responsible for controlling the pituitary gland required neuroanatomical methods that could reveal neurons with a clarity similar to that permitted by the histofluorescence techniques developed in 1962 by Bengt Falck and Nils-Åke Hillarp for catecholamines. Central catecholaminergic neurons were seen for the first time when these methods were applied to the brain (Dahlström & Fuxe 1964). Knowledge of catecholamine structure and chemistry was required to achieve these revolutionary findings. Without knowing the precise structure of the various hypothalamic-releasing factors, it was impossible to develop appropriate visualization techniques for them. Therefore, the reason why the maps changed so little was simple: the techniques that were needed for determining the locations of the hypothalamic neuroendocrine neurons with improved spatial resolution were still unavailable in 1971. But all was to change at the same time that Harris's final review was published posthumously (Harris 1972).

Figure 1

Six maps from 1955 to 2009 showing the hypothalamic locations of regions that contain neuroendocrine neurons and pituitary hormone control mechanisms. They illustrate the dramatic improvement in the resolution of these representations over time. (A) Diagram of a midline sagittal section through the hypothalamus and pituitary gland. The stippled areas indicate the sites where electrical stimulation or lesions resulted in changes of pituitary secretion. ACTH, adrenocorticotrophic hormone; DMH, dorsomedial nucleus of the hypothalamus; LH, luteinizing hormone; MN, mammillary nuclei; PN, posterior nucleus; PVH, paraventricular nucleus of the hypothalamus; TSH, thyroid-stimulating hormone; VMH, ventromedial nucleus of the hypothalamus; OC, optic chiasm; SON, supraoptic nucleus. Reproduced, with permission, from Harris GW (1955*b*) The function of the pituitary stalk. *Bulletin of the Johns Hopkins Hospital* **97** p370, Figure 2. Reprinted with permission of Johns Hopkins University Press. (B) The hypophysiotropic area of the hypothalamus. The solid black line represents the borders of five relatively midline pituitary grafts in which considerable cellular integrity was maintained despite their ectopic site. The dotted circles are the locations of periodic acid-Schiff-positive basophils. AHN, anterior hypothalamic area; AHyp, adenohypophysis; ARH, arcuate nucleus; PM, premammillary nucleus; PP, posterior pituitary; SCH, suprachiasmatic nucleus. Adapted, with permission, from Halasz B, Pupp L & Uhlarik S (1962) Hypophysiotrophic area in the hypothalamus. *Journal of Endocrinology* **25** 147–154. (C) Similar to (A), except both stippling and cross-hatching are used to indicate the sites where electrical stimulation or lesions resulted in changes of pituitary secretion. Reproduced, with permission, from Harris GW (1972) Humours and hormones. *Journal of Endocrinology* **53** ii–xxiii. (D) General diagram of the system of LRF (GnRH)-producing cells in a paramedian sagittal section of guinea pig hypothalamus. The red dots show specifically immunoreactive perikarya. The dotted lines show the pathway of LRF axons (the arrows showing direction of transport). AC, anterior commissure; MM, median mammillary nucleus. Reproduced, with permission, from Barry J, Dubois MP & Poulain P (1973) LRF producing cells of themammalian hypothalamus. A fluorescent antibody study. *Zeitschrift fur Zellforschung und Mikroskopische Anatomie* **146** 351–366. Copyright 1973 Springer-Verlag.

Immunohistochemistry and RIA   By 1971, Guillemin and Schally had determined the chemical structure of the two releasing factors – as Harris argued they should be called (Harris 1972) – for three adenohypophysial hormones: luteinizing hormone (LH), follicle-stimulating hormone (LRF/GnRH), and thyroid-stimulating hormone (TRF/TRH). Those for prolactin (dopamine, a release-inhibiting factor), ACTH (CRF/CRH), and growth hormone (GRF/GHRH and somatostatin) were identified at various times during the following decade. The structures of oxytocin and vasopressin had been worked out in the 1950s (Du Vigneaud 1954–1955). This information quickly led to the generation of specific antibodies, which in turn meant that two methods with effective sensitivity and spatial resolution – immunohistochemistry (IHC) and RIA – could now be used to determine the location of hypothalamic neurons that contain releasing factors and the neural lobe hormones.

Beginning with TRH and GnRH in 1974, Brownstein, Palkovits, and their colleagues used a micropunch technique combined with RIA to document the content of neuroendocrine peptides in hypothalamic and extra-hypothalamic brain regions (Brownstein *et al*. 1974, Palkovits *et al*. 1974, 1976, 1983). These studies were part of a large series of experiments that measured peptides and neurotransmitter content – particularly catecholamines

and their synthetic enzymes – in the brain. Before larger numbers of suitable antibodies for IHC became available in the 1980s, the Palkovits brain micropunch technique provided the highest resolution data for the spatial location of these neurochemicals.

The first immunohistochemical reports of the hypothalamic location of GnRH neurons appeared in 1973 (Barry et al. 1973, Leonardelli et al. 1973). These results immediately and dramatically improved the resolution of hypothalamic maps (Fig. 1D). Over the next 12 years, detailed maps were published showing the brain locations of oxytocin, vasopressin, and all of the releasing factors, with CRH (CRF; Fig. 1E) and GHRH (GRF) being the final ones to be mapped (Swanson et al. 1983, Sawchenko et al. 1985).

Despite the superior spatial resolution of IHC compared to other techniques, it quickly became apparent that the cell bodies of many neuroendocrine neurons contained peptide levels that were below the sensitivity of IHC. The first solution to this problem was to pretreat animals with i.c.v. injections of colchicine to block microtubule formation, which then confined peptides to cell bodies. This widely used method was very helpful for those peptides whose cell body staining was problematic (Lechan & Jackson 1982, Swanson et al. 1983, Sawchenko et al. 1985). But because colchicine is a toxin that interferes with normal neuronal function and structure (Rho & Swanson 1989, Watts 1996), it is difficult to use in experiments that investigate the physiology of neuroendocrine peptides. New approaches were required.

Using gene products and gene manipulations to study neuroendocrine neurons

A huge step forward in studying the location, morphology, and physiological regulation of neuroendocrine neurons began at the end of the 1970s with the cloning of the genes that encode hypothalamic peptide hormones (Roberts et al. 1979). Genes for all of the neuropeptides that are involved with pituitary gland function, along with a host of other neuropeptides, were sequenced during the next decade (see MacLean & Jackson (1988) for a review). This information directly led to the development of two techniques that have dramatically improved our ability to study the physiology of neuroendocrine neurons: in situ hybridization (ISH) in the 1980s; and, later, the transgenic expression of fluorescent and other reporter proteins under the control of neuropeptide gene promoters (Spergel et al. 1999, Young et al. 1999, Herbison et al. 2001).

In situ hybridization

ISH uses radioisotopically or chemically labeled DNA or RNA sequences that are complementary to the RNAs transcribed from peptide genes. This technique can detect mRNAs and the heteronuclear (hn) RNAs that are the primary RNA products of gene transcription (Fig. 2). It has provided enormous insights into the location and control of neuroendocrine neurons during the past 30 years.
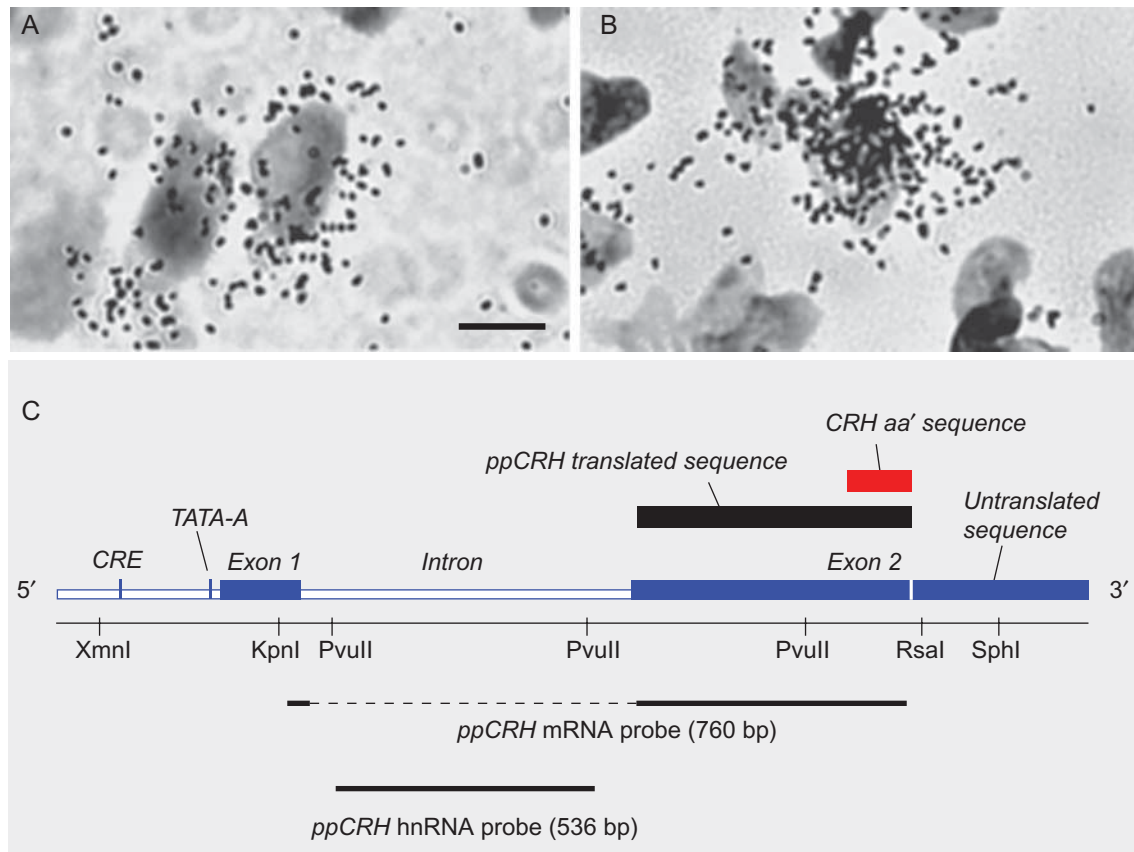
In the first instance, the technique was used to locate those neurons that express the genes for various neuropeptides, particularly for those where IHC could only provide equivocal results (Gee et al. 1983). A flurry of mapping papers using ISH then followed. But the technique was also quickly applied to the investigation of physiologically relevant changes in gene expression, particularly the effects of hormone feedback, physiological challenges, and changes over time (e.g., Young et al. 1986, Koller et al. 1987, Lightman & Young 1988, Zoeller & Young 1988, Swanson & Simmons 1989, Watts & Swanson 1989).

More recently, non-isotopic methods, including colorimetric and, in particular, fluorescent ISH (FISH), have become more popular than radioisotopic ISH. These methods offer greater flexibility for multi-labeling and imaging (e.g., Yue et al. 2008, Babb et al. 2013). But for intact tissue sections, non-isotopic ISH still has difficulty detecting some low-abundance RNAs, such as hnRNAs or the mRNAs for some receptors.

Transgenic expression of reporter proteins

During the 1990s, it became possible to drive the neuronal expression of various reporter genes under the control of specific neuropeptide gene promoters. Although β-galactosidase (encoded by lacZ) has been employed for investigating neuroendocrine neurons (e.g., Schwartz et al. 1998, Skynner et al. 1999), this reporter has largely been superseded in morphological and electrophysiological studies by the transgenic expression of genes that encode fluorescent proteins (FPs) (Spergel et al. 1999, Young et al. 1999, Cowley et al. 2001).

Using specific neuropeptide gene promoters to drive FP gene expression enables the fluorescent labeling of neurons that express a particular peptide gene at some point in their lifetime, often in their entirety. The resultant labeling is permanent and often robust, which mitigates problems with antibody sensitivity. However, it should be remembered that because FP labeling does not directly correlate with peptide or mRNA content at a particular time – indeed, that is why the technique is often so useful as a specific marker – it is not a proxy for the types of quantitative information that can be obtained with IHC or ISH.

As an example of the clarity and spatial resolution that can be provided by transgenic FP labeling, Fig. 3 shows CRH neurons labeled with TdTomato in the paraventricular nucleus of the hypothalamus (PVH) of Crh-IRES-Cre;Ai14
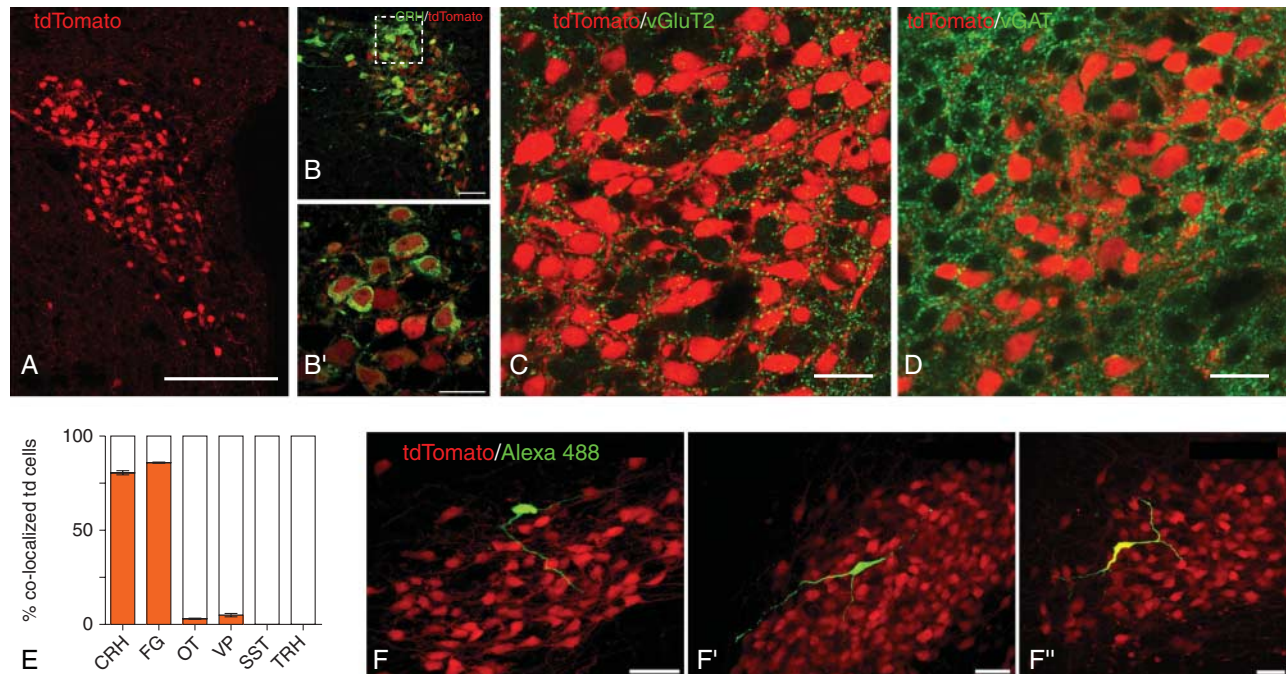
Figure 2

Brightfield photomicrographs of hybridization for CRH mRNA (A) and CRH hnRNA (B) on sections counterstained with thionin to show cell nuclei. Note the cytoplasmic labeling for the mRNA and the nuclear labeling for the hnRNA (scale bar = 5 μm). (C) A schematic representation of the rat pre-procorticotropin-releasing hormone (*ppCRH*) gene. It shows the location of exon 1, the intronic sequence, exon 2, the cyclic AMP response element (CRE), and the TATA-core promoter sequence (TATA-A). Also shown are coding regions for the ppCRH translated sequence (black box) and the CRH amino acid (aa') sequence (red box). The black dashed and solid lines at the bottom of the diagram show the sequences that are targeted by a 760 bp cRNA probe for *ppCRH* mRNA and a 536 bp cRNA probe for *ppCRH* hnRNA that detects the primary transcribed (intronic) sequence. Reproduced, with permission, from Tanimura SM, Sanchez-Watts G & Watts AG (1998) Peptide gene activation, secretion, and steroid feedback during stimulation of rat neuroendocrine corticotropin-releasing hormone neurons. *Endocrinology* **139** 3822–3829. Copyright 1998 The Endocrine Society.

mice (Wamsteeker Cusulin *et al.* 2013). Robust TdTomato labeling is evident only in CRH neurons (Fig. 3A, B, and E), and close appositions can be visualized between VGluT2 (glutamatergic) or vGAT (GABAergic) elements and CRH neurons (Fig. 3C and D). FP-labeled CRH neurons are easily sampled for electrophysiological recording (Fig. 3F). In addition, they can be optogenetically manipulated by the viral Cre-driven expression of channelrhodopsin (Wamsteeker Cusulin *et al.* 2013). For other neuro-endocrine peptides, transgenic expression of FPs has been used very effectively to examine the connections, electro-physiology, and morphology of GnRH neurons (Suter *et al.* 2000, Herbison *et al.* 2001, Campbell & Herbison 2007, Iremonger *et al.* 2010) and to identify the neurotransmitter phenotypes of POMC neurons in the arcuate nucleus (Hentges *et al.* 2009).

Mapping and neuroinfomatic techniques The first atlases of the hypothalamus were published in the 1930s (Krieg 1932, Le Gros Clark 1936, Rioch *et al.* 1940). These remained definitive well into the 1950s, and they clearly influenced the maps that were produced by Harris and Halasz (Fig. 1A, B, and C). But even a cursory glance at any examples of spatial representation of neuroendocrine function from that time shows that these locations were projected onto what are rather rudimentary maps. More accurate atlases started to emerge in the 1950s and 1960s (de Groot 1959, Christ 1969), and these helped support increasingly accurate maps of neuroendocrine topography in the hypothalamus (Fig. 1D and E).

As greater amounts of high-resolution spatial data were generated from IHC, ISH, and FP expression, atlases with equivalent accuracy became required so that

Figure 3

Anatomical distribution and CRH protein expression in tdTomato-labeled cells in the paraventricular nucleus of the hypothalamus (PVH) of *Crh-IRES-Cre;Ai14* mice. (A) Confocal image (20× magnification) of the PVH in a *Crh-IRES-Cre;Ai14* (tdTomato, red) mouse (level 61 of Dong (2007)). (B) Confocal image (40×) of a colchicine-treated *Crh-IRES-Cre;Ai14* mouse PVN. Immunostaining for corticotropin-releasing hormone (CRH) is shown in green. (B′) Higher magnification (100×) of the box inset in (B). Confocal images of the PVH in *Crh-IRES-Cre;Ai14* (tdTomato, red) mouse labeled for vGluT2 (C, green), or vGAT (D, green). Note the numerous close appositions (yellow) between tdTomato (CRH) neurons and glutamaterigic (vGlutT2) and GABAergic (vGAT) elements. (E) Bar graph showing the percentage of tdTomato-positive cells that co-express various neuropeptides in five colchicine-treated mice. CRH, corticotropin-releasing hormone;

FG, fluorogold; OT, oxytocin; SST, somatostatin; TRH, thyrotropin-releasing hormone. (F) Confocal images (60× magnification) from three mouse PVH sections (F, F′, and F″) showing the morphology of single tdTomato neurons (red) filled with Alexa488 (green) and biocytin (yellow) during whole-cell patch clamp recordings. Scale bars are 100 μm (A), 50 μm (B, F, F′, and F″), and 20 μm (B′, C, and D). (A, B, E, and F) reproduced from Wamsteeker Cusulin JI, Füzesi T, Watts AG & Bains JS (2013) Characterization of corticotropin-releasing hormone neurons in the paraventricular nucleus of the hypothalamus of Crh-IRES-Cre mutant mice. *PLoS ONE* **8** e64943. Published under the Creative Commons Attribution (CC BY) license. (C and D) Unpublished photomicrographs from the author's laboratory (CS Johnson & AG Watts).
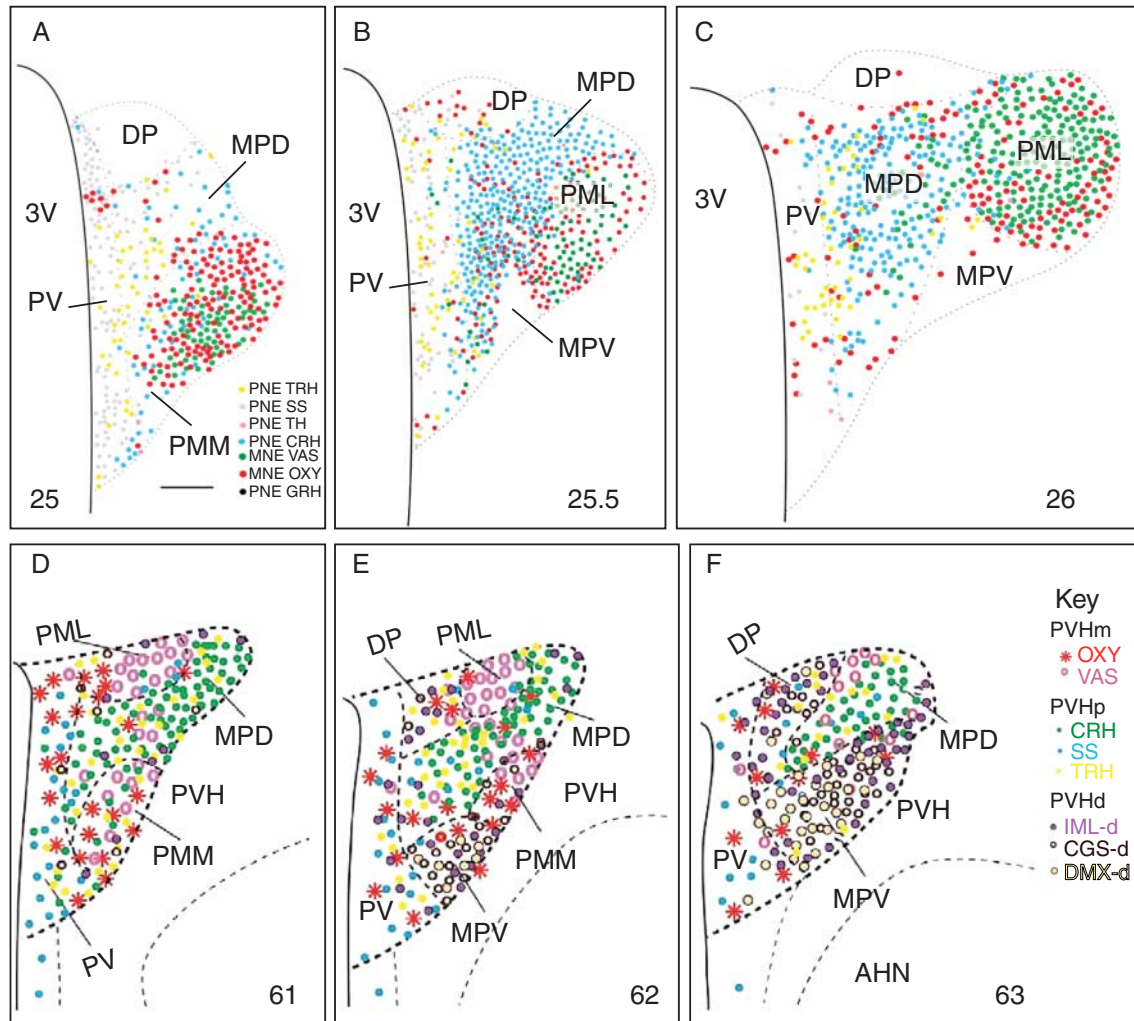
investigators could take full advantage of their new findings. They started to be produced in the early 1980s when new rat and mouse brain atlases began to be published (Paxinos & Watson 1982, Swanson 1992, 2003, Franklin & Paxinos 1996, Dong 2007). These atlases made it possible to produce high-precision topographies of neuroendocrine neurons. Figures 1F and 4 show recent topographies of the neuroendocrine PVH in the rat and mouse using maps from these atlases (Simmons & Swanson 2009, Biag *et al*. 2012, Watts & Khan 2013).

The importance of using accurate maps now goes further than simply facilitating topographic analyses. Neuroinformatics provides new and powerful tools for analyzing the highly complex brain connectional and topographic data that is now being generated (e.g., Zingg *et al*. 2014, Bota *et al*. 2015). This is making it increasingly important to represent data on accurate and, of particular

importance, standard and widely available – often online – brain atlases. Accurate topographies greatly facilitate data input into databases, atlases, and neuroinformatics tools. But critically, they also enable neuroanatomical data to be directly compared between different experiments, experimenters, labs, etc. This is a central feature of experimental science, and it is one that is much more difficult to achieve with the types of unique, single investigator-generated maps that are sometimes used to present results.

The pituitary stalk as a final common motor pathway to the pituitary gland

Neuroendocrine neurons as motor neurons   An important step toward investigating how any neural system is organized is to establish a conceptual framework as the basis for structural and functional experiments. For the

Figure 4

(A, B, and C) Three maps of the rat PVH taken from a reference atlas of neuroendocrine neuron type labeled with antibodies for neuropeptides. The locations of each type of peptidergic neuroendocrine neuron are plotted onto three levels of PVH (designated by the number at the bottom of each panel) (data from Swanson 2003, Simmons & Swanson 2009). (D, E, and F) Schematic drawings illustrating the delineations of three levels of the PVH in the mouse brain, which were determined based on the distributions of eight neuronal phenotypes in two major neuroendocrine divisions (magnocellular (m) PVHm, including OXY and VAS; and parvicellular (p) PVHp, including CRH, SS, and TRH) and three descending preautonomic populations (PVHd) that project to the intermediolateral column of the spinal cord (IML-d), to the central gray of the spinal cord (CGS-d), and to the dorsal motor nucleus of the vagus nerve (DMX-d). The mouse atlas levels (Dong 2007) are designated by the number at the bottom of each panel.

Reproduced, with permission, from Biag J, Huang Y, Gou L, Hintiryan H, Askarinam A, Hahn JD, Toga AW & Dong HW (2012) Cyto- and chemoarchitecture of the hypothalamic paraventricular nucleus in the C57BL/6J male mouse: a study of immunostaining and multiple fluorescent tract tracing. *Journal of Comparative Neurology* **520** 6–33. Copyright 2011 Wiley Periodicals, Inc. 3V, third ventricle; AHN, anterior hypothalamic nucleus; CRH, corticotropin-releasing hormone; DP, dorsal parvicellular part of the PVH (descending division); GRH, growth hormone-releasing hormone; MNE, magnocellular neuroendocrine; MPD, medial parvicellular part of the PVH, dorsal zone; MPV, medial parvicellular part of the PVH, ventral zone; OXY, oxytocin; PML, posterior magnocellular part of the PVH, lateral zone; PMM, posterior magnocellular part of the PVH, medial zone; PNE, parvicellular neuroendocrine; PV, periventricular part of the PVH; SS, somatostatin; TRH, thyrotropin-releasing hormone; VAS, vasopressin.

neuroendocrine system, a sound framework of this nature took a long time to develop. Reports in the 1920s and 1930s that showed the existence of 'nerve-gland cells' in the hypothalamus of many vertebrate species (Scharrer & Scharrer 1940) came from efforts to explain how environmental stimuli could alter 'internal secretions'

(Watts 2011). The result of these investigations was neurosecretion.

During the 1930s and 1940s, neurosecretion was a concept that had numerous flaws and inconsistencies (Watts 2011). It was unable to make any meaningful impact on understanding how the hypothalamus

controls the pituitary gland until about 1950, when the results of Palay (1945) and Bargmann (1949) were assimilated into the field (Watts 2011). Nevertheless, the emphasis on explaining how sensory information controls the pituitary gland remained an essential theme for the entire field and showed that projections from many parts of the brain to the hypothalamus must at some point influence neuroendocrine neuronal function. Results from experiments in the 1950s showed that interosensory and extrasensory information converges on integrative mechanisms in the hypothalamus, which then directly controls hormone release from the pituitary gland (e.g., Sayers *et al*. 1958).

Moving the field forward with novel structural and functional experiments required a conceptual framework to explain how this control occurred. To this end, in 1955, Harris (1955*b*) compared neuroendocrine control to the classic voluntary motor control system that had been established during the previous 100 years.

Harris & Fortier (1954) proposed that the pituitary stalk formed the connecting link:

'between the external environment and the central nervous system on the one hand, and the pituitary gland and its target organs on the other'.

Harris (1955*b*, p 371)

He then likened sensorimotor integration in the cortex (i.e., the representation of different parts of the body in the sensory and motor cortex) to the locations of the different mechanisms that are responsible for controlling the secretion of the various pituitary hormones. Harris continued this line of thinking by noting the similarity of the pituitary stalk to the ventral horn of the spinal cord. A logical extension of this comparison was to say that the supraopticohypophysial tract (and most likely the hypophysial portal vessels) is a final common pathway – in the Sherringtonian sense – that is used by the brain to control hormone release from the pituitary gland (Harris 1955*b*, p 360). Harris therefore believed that the function of the hypothalamic neurons that control the posterior pituitary is motor in nature. He made the explicit comparison between the posterior pituitary and a voluntary muscle, stating that:

'both structures are dependent on their innervation for any functional activity, even to the extent that they undergo atrophy if denervated'.

Harris (1955*b*, p 359)

Therefore, the basic premise of Harris's discussion was that the neuroendocrine neurons that are responsible for controlling pituitary hormone release are, in principle, motor neurons like those in the ventral horn of the spinal cord: both directly control the activity of an organ located outside the brain.

One prediction of this model is that neuroendocrine control mechanisms are organized similarly to voluntary movement and autonomic motor control (Saper 2002, Thompson & Swanson 2003). In this way, neuroendocrine motor neurons are directly controlled by premotor neurons and pattern generators, which in turn are influenced by a comprehensive set of inputs that allow many and varied interosensory, exterosensory, and emotional influences to control pituitary hormone release.

Harris does not seem to have pursued the implications of his comparison any further, and it does not appear to have been picked up to any extent by others in the field during his lifetime. As we saw in the section Spatial localization of function in the neuroendocrine hypothalamus on the localization of function, this was not surprising because sufficient details about the nature, morphology, location, and hodology of hypothalamic neuroendocrine neurons were still unknown when Harris died in 1971. Although a general understanding of the complex inputs from the rest of the brain to the hypothalamus that control ACTH secretion, for example, was already reasonably well developed in the late 1950s (Sayers *et al*. 1958), many of the other key structural elements of neuroendocrine motor control still needed to be elucidated. Since 1971, however, a multitude of studies have characterized these features in great detail, which has thereby allowed the development of more extensive conceptual models of neuroendocrine control based on Harris's original premise (e.g., Watts & Swanson 2002, Thompson & Swanson 2003, Watts 2005, Watts & Khan 2013).

Premotor control networks as hormone release pattern generators It has been known for decades that LH secretion is comprised of two modes: a pulsatile (or episodic) pattern that is most apparent during basal conditions; and a surge pattern that is seen during stimulation and is superimposed upon pulsatile release. Although all pituitary hormones show these two patterns to a greater or lesser degree (with the nature and timing of the patterns varying from hormone to hormone), the basic organization is nevertheless maintained (Watts 2005).

If a model of neuroendocrine control based on voluntary movement and autonomic motor control is tenable, then we should be able to identify distinct sets of

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premotor neurons that are responsible for each of these two release patterns, along with direct connections between each of the different components. For most neuroendocrine neurons, this continues to be extremely difficult, not least because directly measuring their output into hypophysial portal blood in response to experimental manipulations remains a considerable technical challenge. Furthermore, neuroanatomical tracing techniques for identifying projections to and from a single neuronal cell type have, until very recently, been unavailable.

Support for the idea that neuroendocrine neurons are controlled by premotor neurons and motor pattern generators (or analogues thereof) has perhaps been best developed for GnRH neurons. The fact that immortalized GnRH neurons organize themselves in a manner that supports pulsatile release in the absence of all other inputs (Wetsel *et al*. 1992) is consistent with the idea that pulsatility is the fundamental release pattern of these particular neuroendocrine motor neurons. In whole animals, pulsatile release from GnRH neurons (and perhaps all types of neuroendocrine neurons) is then modified by various premotor inputs into more complex surge patterns. In turn, the actions of these premotor neurons are further shaped by their own particular sets of inputs (Watts 2005).

The investigation of the structural and functional organization of the GnRH control networks was made much clearer when kisspeptin was identified as a potent activator of GnRH neurons and as an essential component for the onset of puberty (reviewed by Dungan *et al*. (2006)). Kisspeptin neurons were then found in the rodent anteroventral periventricular (AVPV) and arcuate (ARH) nuclei (Lehman *et al*. 2010). This arrangement is broadly maintained across diverse mammalian species (Goodman & Lehman 2012). Kisspeptin strongly stimulates the firing rate of GnRH neurons (reviewed by Piet *et al*. (2015)), and the two populations of kisspeptin neurons directly innervate or have some form of interaction with GnRH neurons (Clarkson & Herbison 2006, Lehman *et al*. 2010). These two kisspeptin populations are implicated in the direct control of either the pulsatile (ARH) or the surge (AVPV) release modes (reviewed by Piet *et al*. (2015)), and both are required for normal surge activity and estrous cyclicity (Hu *et al*. 2015).

Much of the detailed hodology of kisspeptin neurons remains to be determined, but the fact that the LH surge is superimposed on the more fundamental pulsatile release (Fox & Smith 1985, Hoeger *et al*. 1999) would require connections from the AVPV kisspeptin to the ARH

kisspeptin populations. Although projections of this type between these two nuclei have been shown to exist (Gu & Simerly 1997) and to involve kisspeptin AVPV neurons (Yeo & Herbison 2011), whether there are direct projections from AVPV kisspeptin to ARH kisspeptin neurons remains to be determined (Lehman *et al*. 2010).

Future challenges

Chemical neurotransmission and neuroendocrine motor neurons

The vast majority of our current understanding about brain function rests on the foundation of chemical neurotransmission at the synapse, which is a rapid and spatially restricted means of communication. As the fundamentals of neuroendocrinology emerged in the 1930s and 1940s, neurosecretory systems were thought to communicate in ways that were different from other parts of the brain. At that time, signaling between conventional neurons was believed to be electrically mediated (Watts 2011). But as neuroscientific concepts inundated neuroendocrinology, it became clear that neuroendocrine neurons function in basically the same way as conventional neurons do in terms of the ionic basis of action potential propagation, vesicular release of chemical signals, etc. (Watts 2011). As the fine structure of neuroendocrine neurons has been determined with increasing detail, new facets of neuroendocrine communication have emerged. One example is the release of neuropeptides from the dendrites of neuroendocrine neurons, which was first identified by electron microscopy about 25 years ago (Pow & Morris 1989). Recent work from Stern and his colleagues now shows that the dendritic release of vasopressin provides a way for magnocellular neuroendocrine neurons in the PVH to modify the activity of nearby preautonomic neurons, which thereby mediates the integration of these two functionally distinct compartments (Son *et al*. 2013, Stern 2014).

Conventional neural projections provide obvious ways for integrating the functions of different control systems. But as dendritic release and other forms of more spatially diffuse transmission show (Fuxe *et al*. 2007), non-synaptic release mechanisms likely play a significant role in these integrative processes. The way in which these types of chemical signaling work together with synaptic neurotransmission to effect neuroendocrine integration offers an intriguing new way of considering neuroendocrine motor control functions.

The spatial and hodological organization of the neuroendocrine hypothalamus

We are still far from knowing how the neuroendocrine hypothalamus is organized with the same clarity that is currently available for a system such as the hippocampus. The topological heterogeneity of neuroendocrine motor neurons (Fig. 4) along with the great complexity of the connections into the neuroendocrine hypothalamus have hampered efforts to probe the detailed organization of the control networks.

Conventional tracing techniques have been used for the past 45 years to examine hypothalamic connectivity, and they have provided a very useful framework (Watts & Swanson 2002). But they do not have sufficient cell specificity to determine whether neuroendocrine control networks really are organized with the same principles as those that control voluntary movement or, in particular, autonomic output; the neuroendocrine motor system may have close organizational relationships with the later networks. The organization of premotor networks for GnRH neurons is better known than that of premotor networks for other adenohypophysial-releasing hormones. But details remain inadequate for describing the overall structure of these premotor networks and for assessing how they fit into the larger integrative networks that are distributed throughout the brain and that coordinate neuroendocrine and autonomic motor actions with motivated behaviors.

A major way forward for revealing these interactions comes from the ability to label single-neuron populations with viruses that can drive the expression of FPs. These methods are now providing opportunities for characterizing the hodology of phenotypically defined neuronal populations (Krashes *et al.* 2014, Sun *et al.* 2014), and the should prove extremely useful for regions that contain mixed populations of neuroendocrine neurons, such as the PVH (Fig. 4).

Conclusion

The mid-1950s was a period of considerable debate about how the brain controls the different parts of the pituitary gland. Although evidence was already leaning heavily toward the view that the pituitary stalk contains the axons that transport oxytocin and vasopressin from the hypothalamus to the posterior pituitary (and most likely also transmit the nerve impulses that release them), how signals from the hypothalamus control the adenohypophysis was still hotly contested. The experiments performed by Geoffrey Harris during the previous 8 years with John Green, Dora Jacobsohn, and others continued to strengthen the neurohumoral transmission hypothesis that had first been proposed by Hinsey and Markee almost 20 years earlier (Hinsey 1937). But it was a hypothesis that was still far from being universally accepted. And so it was in the context of this debate that Harris contributed a short review in 1955 that presented a new framework for the neuroendocrine control of the adenohypophysis (Harris 1955*b*). This review argues that Harris's framework was built solidly on the rapidly developing neuroanatomy and neurobiology of the time and was perhaps an early sign that neuroendocrinology could dovetail with the then embryonic discipline of neuroscience. The large amount of work on neuroendocrine anatomy in the 60 years since the publication of Harris's review has clearly vindicated his foresight about the structural bases of neuroendocrinology.

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