CHAPTER 1

The Framework Upon Which Current Research on the GnRH Neuron and its Control is Built

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Abstract

The ovarian cycle and ovulation in the female, and spermatogenesis in the male, are dependent upon the brain, and specifically upon a diffusely distributed network of peptidergic neurons in the hypothalamus that synthesize the neurohormone, gonadotropin-releasing hormone (GnRH). The decapeptide was isolated in 1971 from bovine and ovine hypothalami by the laboratories of Andrew Schally and Roger Guillemin, respectively (Matsuo et al., 1971; Amoss et al., 1971), and initially termed luteinizing hormone releasing hormone (LH-RH) or luteinizing hormone releasing factor (LRF). This review provides a brief historical account of the development of the concepts underpinning current research on the GnRH neuron and its control.

1.1 Introduction

The idea that the gonads might be governed by the central nervous system (CNS) via a neurohormone had emerged well before the isolation of GnRH. It had been apparent for centuries that reproduction is closely related to environmental cues. For example, sheep in the northern hemisphere generally breed during the months of September–November, but if these animals are relocated to the southern hemisphere, a 180° phase shift in this behavior occurs and breeding is observed from February–April. This and other observations were taken by F.H.A. Marshall, at the

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University of Cambridge, to champion the view during the first half of the 20th century that reproduction was governed by the CNS. While Marshall was formulating the ideas of the CNS control of reproduction, the foundations of endocrinology were also being laid. In the present context, it was established that extracts of the anterior pituitary, now known to contain the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), were able to stimulate the gonads. The nerve supply of the ovary and testis, on the other hand, was recognized as scant and limited primarily to vasomotor control. These two views naturally led to the idea that environmental cues, such as those that regulate seasonal breeding, were registered by the CNS and relayed to the gonads by hormonal signals from the pituitary. That the hypothalamus was the critical region of the brain in the regulation of pituitary function was established between 1925 and 1950 by classic physiological experimentation using lesions, electrical stimulation, and pituitary transplantation (see Harris, 1955).

The question of how the hypothalamus communicates with the anterior pituitary was heatedly debated during the 1940s and early 50s. One school, championed by Geoffrey Harris, proposed a humoral link utilizing the specialized portal vasculature between the hypothalamus and anterior pituitary. A second, led by Solly Zuckerman, focused on the more traditional pathway used for control by the CNS; that is, a direct neural link. For an account of this important era of neuroendocrine research, the reader is referred to the classic monograph written by Harris, entitled "The Neural Control of the Pituitary Gland" (Harris, 1955), where he reiterated his view, developed earlier in collaboration with J.D. Green, "that nerve fibres of the hypothalamus liberate some humoral substance into the primary plexus of the hypophysial vessels and that these vessels transmit the substance to the adenohypohysis where it exerts an activating effect on the gland cells." However, it was not until 1971, with the isolation and characterization of GnRH, that the neuro-humoral hypothesis for the control of the gonadotropin secreting cells of the anterior pituitary was finally accepted by all. The last act in the discovery of GnRH involved a protracted "race" between the laboratories of Guillemin and Schally to isolate the gonadotropin releasing neurohormone from the hypothalamus. This race was at times acrimonious, although its successful conclusion, together with the earlier isolation of thyrotropin releasing hormone (TRH), was responsible for the award to these two scientists of half the Nobel Prize for Physiology or Medicine in 1977 (the other half was awarded to Ross Yalow for "the development of radioimmunoassays of peptide hormones"; see later). A riveting account of the discovery of GnRH has been provided in a series of three articles written by Nicolas Wade and published in *Science* in April/May 1978.

1.2 Approaches taken to study the GnRH neuron

Two fundamental approaches were taken to understand the GnRH neuron and its control following the isolation and characterization of GnRH in 1971. The first was a direct "neurobiological" approach, which employed anatomical, cellular, and electrophysiological methodologies that examined the location and morphology, birth, migration, and cellular and molecular biology of the GnRH neuron. The second was an indirect "endocrinological" approach that treated the GnRH neuron as a "black box" and investigated its regulation and output (tracked indirectly by measuring LH in peripheral blood) in the context of the control system that governs various aspects of gonadal function. It is worth noting that both approaches greatly benefited from an appreciation of immunology that enabled specific antibodies to be generated against GnRH and LH, which were then used to develop sensitive immunohistochemical (IHC) procedures and radioimmunoassays (RIAs) for these two peptide hormones.

1.2.1 Neurobiological approach

The IHC localization of hypothalamic GnRH, pioneered by Julien Barry and his colleagues in Lille, France in the early 1970s (Barry et al., 1973), soon revealed three important features of GnRH neurons in the mammalian hypothlamus: first, there were only a few hundred of these peptide neurons; second, they were diffusely distributed throughout the hypothalamus; and third, their far-reaching projections were striking. The diffuse distribution of GnRH neurons in the hypothalamus is a characteristic that, to this date, has frustrated cellular investigations of these cells, including those relating their electrophysiological properties to their secretory activity. Later IHC studies of GnRH neuron location in the embryonic brain by Donald Pfaff and Susan Wray led to the recognition of another peculiar feature of the GnRH neuron, namely that unlike other neurons, it is not born in the ependymal lining of the cerebral ventricles, but rather outside the brain in the nasal placode (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). This means that before the GnRH neuron can subserve a hypophysiotropic function, it has to enter the brain and migrate through the forebrain to the hypothalamus: a complex process that takes place during early embryonic development. Most recently, contemporary transgenic, electrophysiology, and imaging techniques have led to the view that projections from the GnRH cell body to the median eminence, where the primary plexus of the hypophysial portal circulation is located, exhibit the unique feature of possessing properties of both axons and dendrites; these projections are now termed "dendrons" (Herde et al., 2014).

Parenthetically, the GnRH gene (*GnRH1*) was cloned from human and rat by the Seeburg laboratory in the mid-1980s, and our understanding of the regulation of expression of *GnRH1* was greatly facilitated by the creation of an immortalized GnRH cell line from the mouse brain using targeted tumorigenesis (Mellon et al., 1990).

1.2.2 Endocrinological approaches

The endocrinological approach has invariably involved studies of the female, because ovulation is a key and easily identifiable event of the ovarian cycle, and, historically, one that provided the only reliable surrogate marker of acute hypothalamic activation (i.e., GnRH discharges). Application during the early 1970s of LH and FSH RIAs to various species, particularly the monkey and human, had indicated that the pattern of gonadotropin secretion during the ovarian cycle could be conceptualized as comprising two modes of secretion: a basal or tonic mode, observed during the follicular and luteal phases of the cycle, which was interrupted at mid-cycle by an abrupt and large discharge or surge of LH and FSH, known as the pre-ovulatory gonadotropin surge; this was what was responsible for ovulation.

Before the structure of GnRH was reported, Ernst Knobil and his colleagues in Pittsburgh had observed that circulating LH concentrations measured at frequent intervals exhibited a striking saw-tooth pattern with peak levels at approximately hourly intervals in the ovariectomized rhesus monkey (Dierschke et al., 1970). They proposed that this pulsatile or episodic mode of gonadotropin secretion is likely due to intermittent signals from the brain that are relayed to the anterior pituitary by an "LRF." It was not until 1982, however, that the pulsatile mode of this LRF (i.e., GnRH) release into the portal circulation was empirically demonstrated by the group of Iain Clarke (Clarke and Cummins, 1982). By this time, the notion that an intermittent pattern of GnRH stimulation was required to sustain gonadotropin secretion had become dogma: in 1978, Knobil's laboratory reported that, in GnRH-deficient monkeys, gonadotropin secretion could only be maintained when exogenous GnRH was administered as brief pulses at approximately hourly intervals (Belchetz et al., 1978). The foregoing studies of Knobil, and contemporaneous work by Fred Karsch's laboratory investigating the neuroendocrine basis of seasonal breeding in sheep, led to the idea of a hypothalamic "pulse generator" responsible for the intermittent release of GnRH, which in turn drives pulsatile gonadotropin secretion (Goodman et al., 1981; Pohl and Knobil, 1982): a concept that has become a cornerstone of the neuroendocrine control system governing ovarian and testicular function in the adult.

Interestingly, in higher primates, including humans, robust pulse generator activity, as reflected by LH secretion, is apparent during mid-fetal

development and again during infancy (i.e., several years before puberty: intuitively, the stage of development when initiation of pulsatile GnRH release might be anticipated). This perinatal time course in LH secretion led to the concept that the quiescence of the pituitary–gonadal axis of the child and juvenile is occasioned by a developmental suppression of the GnRH pulse generator and that puberty is a reflection of a re-augmentation of pulse generation (see Plant, 2015).

1.2.3 Integrative approaches

The origins of the concept that the ovarian cycle was regulated by feedback signals from the ovary were laid in the early 1930s, and it later emerged that estradiol 17beta (E2) was a major component of these feedback signals. Two feedback actions of ovarian E2 were recognized: a negative feeback action that was involved in regulating tonic gonadotropin secretion, and a positive feedback action that was responsible for triggering the pre-ovulatory LH surge at the end of the follicular phase of the ovarian cycle. As understanding of the neurovascular control of anterior pituitary function evolved, it became clear that ovarian E2 must regulate gonadotropin secretion by either a direct action on the pituitary or an indirect action on the brain (to control GnRH release), or by a combination of the two.

With respect to the feedback actions of E2 at the brain, the most parsimonious hypothesis underlying a mechanism of action of this steroid to regulate GnRH secretion was that the target of the steroid was the GnRH neuron itself. However, application of IHC coupled with contemporary gene knockout strategies has led to the current consensus that this is not the case (see Herbison, 2015). Indirect control of the GnRH neuron seems to be the predominant mechanism of regulation utilized by most modulators of this hypothalamic cell type, including developmental cues, seasonal signals, stress, and metabolic and nutritional factors. Recognition of such upstream control of the GnRH neuron has led over the years to a concerted effort to identify the proximal signals regulating GnRH release, initially employing pharmacological approaches, and more recently gene knockout strategies. Typical of this era of research was the individual laboratory focused on elucidating the contribution of a “favorite” neurotransmitter, neuropeptide, or glial factor as a proximal signal controlling GnRH release.

The foregoing idiosyncratic approach changed dramatically following two almost simultaneous and independent clinical reports – one by Seminara et al. (2003) (a joint venture between a group in Boston and two in Cambridge) and one by de Roux et al. (2003) in Paris – that loss-of-function mutations of G protein coupled receptor 54 (GPR54) were associated with hypogonadotropic hypogonadism and delayed or absent

puberty. The Boston group also demonstrated that the pituitary of these subjects secreted LH in response to exogenous GnRH, indicating that the impact of the receptor mutation was manifest at a supra-pituitary level, presumably at the hypothalamus. Prior to 2003, activation of GPR54 signaling by the receptor's ligand, metastin, was recognized to suppress metastasis in certain models, but, remarkably, the neuroendocrine community had no inkling that this signaling pathway was involved in the regulation of the pituitary–gonadal axis. It soon became apparent, however, that metastin (now termed "kisspeptin" by the neuroendocrine community) was an exceptionally potent GnRH secretogog and that GPR54 (a.k.a. kisspeptin receptor 1, KISS1R) was expressed by the GnRH neuron. Together, these findings led to the present kisspeptinocentric era of research on the GnRH neuron and its control: the overwhelming majority of investigators studying the neuroendocrine control of the gonad, regardless of what modality of GnRH secretion they are interested in, and irrespective of the animal models they employ, have become consumed by the role of kisspeptin in governing the GnRH neuron.

This approach was reinforced 6 years later by two events. First, further application of contemporary human genetics revealed that loss-of-function mutations in another peptide signaling system, neurokininB (NKB) and its receptor (TACR3), were associated with hypogonadotropic hypogonadism and delayed or absent puberty (Topaloglu et al., 2009); a phenotype very similar to that reported in 2003 for mutated GPR54. Second, it was recalled that NKB was expressed in the same neurons in the arcuate nucleus that express kisspeptin. Thus, the neuroendocrine community was faced with the fascinating idea that two neuropeptides, each of which appeared essential for puberty and subsequent gonadal function, were expressed in the same hypothalamic neurons. This realization, together with the finding that in many species these arcuate neurons also express dynorphin, a peptide inhibitory to GnRH release, was the impetus for the development over the last few years of a compelling neurobiological model for the GnRH pulse generator, now termed the "KNDy hypothesis" (see Lehman et al., 2010).

The idea that ovulation was governed by a neural signal had been generally accepted since the classical studies by John Everett and Charles Sawyer in Los Angeles in the 1940s demonstrating that at a specific time during the 24-hour light–dark cycle, known as the "critical" period, the brain of the female rat generated a recurring daily neural signal that on the day of proestrus was relayed to the pituitary to trigger the preovulatory gonadotropin suge (Everett and Sawyer, 1950). That this daily neural signal originated in the preoptic area (POA) of the hypothalamus was subsequently demonstrated in Sawyer's department at the University of California at Los Angeles by Bela Halász and Roger Gorski, who

used a bayonet-shaped knife (the Halász knife) to surgically isolate ("deafferentate") the medial basal hypothalamus (MBH) from the more anterior region of the forebrain: a procedure that blocked ovulation in the rat (Halász and Gorski, 1967). In 1976, George Fink's laboratory in Oxford provided conclusive evidence for the proestrus neural signal by directly demonstrating that a large discharge of GnRH in hypophysial portal blood coincided with the pre-ovulatory LH surge in the rat (Sarkar et al., 1976). Shortly thereafter, Robert Goodman, a graduate student in Knobil's laboratory, presented compelling evidence that, in the rat, the POA was the critical site of action for the positive feedback effect of E2 (Goodman, 1978). Contemporary work indicates that the E2 responsive neurons in the POA of rodents are located in the rostral periventricular area of the third ventricle (RP3V), and that kisspeptin neurons in this region have all the attributes of a neuronal phenotype for mediating the positive feedback of E2 in these species (Herbison, 2015).

That the foregoing classical view of the neural control of ovulation may not apply to all spontaneously ovulating species emerged from the Knobil laboratory during the 1970s. Deafferentation of the MBH in the monkey, in contrast to the rat, did not consistently interrupt ovulation (Krey et al., 1974), and ovulatory menstrual cycles could unfold in GnRH-deficient hypothalamic lesioned monkeys receiving an invariant intermitent GnRH replacement regime (Knobil et al., 1980). The latter finding led Knobil to posit that the role of the hypothalamus in driving the ovarian cycle in primates was permissive and that the positive feedback action of estradiol in these species was exerted at the pituitary. This idea was never fully accepted by the neuroendocrine community, and recent observations that E2 responsive kisspeptin neurons are also found in an area of the primate POA analogous to the RP3V of rodents have again rekindled the long-standing debate over the relative importance of brain vs. pituitary sites for the positive feedback action of E2 that triggers the pre-ovulatory LH.

1.3 The future

In the chapters that follow, the reader will be able to glimpse at what the immediate future of research on this brain cell likely holds. Today's GnRH scientist has at hand a powerful armamentarium that would have been unimaginable to Geoffrey Harris and his contemporaries of the 1940–50s, and it may be anticipated that answers to several longstanding and fundamentally important questions will be forthcoming. In this regard, the mechanism responsible for the onset of puberty and the precise nature of the neurobiological circuitry underlying GnRH pulse generation spring to mind. Optimism for the future of the field must be tempered by the fiscal

restraints currently facing academic biomedical research in the Western world, and by the strengthening socio-political climate that views animal research, and particularly that on primates, with negative connotations. Our understanding of the GnRH neuron and its control is founded upon studies of multiple species, a theme that resonates in the chapters of this Masterclass: sheep and goats provide models of choice for the direct measurement of the output of the GnRH neuron; mice are invaluable for questions that could not be convincingly answered without transgenic manipulations, and primates are an excellent model for studies of the menstrual cycle and human puberty. Moreover, species differences in the hypothalamic regulation of the gonads have fostered exciting and sustained scientific dialog, thereby enriching the field. Thus, the extent to which this comparative approach is maintained in the future will likely dictate the success of our attempts to obtain a truly comprehensive understanding of the GnRH neuron and its control.

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