

Hypothalamic Control of Anterior Pituitary Function: A History

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The concept of neurohumoral control of anterior pituitary function championed by Geoffrey Harris was based upon clinical and biological observation backed by rigorous experimental testing. The areas of the brain involved in the control of gonadotrophic hormone synthesis and release were identified by electrical stimulation, lesioning and fibre tract cutting. The medial pre-optic area (MPOA) proved to be a major integrating centre, with axon terminals from this region terminating at the median eminence releasing factors into the portal vessels to give a direct route from brain to pituitary. It took over a decade before the gonadotrophic hormone-releasing hormone (GnRH) was isolated, sequenced and synthesised. With antibodies raised against this peptide, the MPOA was identified as a site rich in GnRH neurones and the hormone was detected at high levels in portal blood extracts. A natural knockout of the GnRH gene was discovered in a hypogonadal (hpg) mouse. Hormone injections, gene replacement methods and neural grafting in these mutants all confirmed the central role of GnRH in reproduction. The modern techniques of molecular biology have allowed us to extend our knowledge base. In the last few years the role of kisspeptin and its receptor (GPR54) in the control of the GnRH neurone has added a further level of hypothalamic involvement in the modulation of reproduction.

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Many of our basic concepts in biomedical research were formulated from ideas based upon clinical observation or animal experimentation and further modified over time as technology allowed more rigorous measurement and analysis. In endocrine-related research this has involved surgical manipulation, the extraction and characterisation of bioactive molecules, and their assay in tissues and body fluids. The association of acromegaly with pituitary tumours and the stimulation of body growth in rats following the injection of pituitary extracts in the early 1900s exemplify how clinical observation and laboratory investigation can complement each other. Surgical techniques and expertise were crucial in elucidating the functions of the anterior pituitary gland. The parapharyngeal approach to removal of the gland in rats pioneered by Smith (1) resulted in gonadal, thyroid and adrenal atrophy, and a cessation of body growth. Daily subcutaneous grafts of pituitary tissue reversed these effects. At that time, methods for extracting, purifying and characterising the structure of hormones were in their infancy; nevertheless, the main cornerstone of endocrine research, removal of a tissue and the effects of replacement therapy were established.

This short review will concentrate mainly on hypothalamic control of pituitary gonadotrophic hormone secretion covering the early period in which the so-called releasing factors were isolated

and characterised and comparing the methodology available at that time with the enormous strides in technology that have opened up a whole new level of neural control in the past 5 years or so.

The observation that many animal species demonstrate some degree of seasonal breeding, and that ovulation in a variety of species depends upon a mating-induced reflex release of gonadotrophic hormones, provided evidence that the brain can transduce external signals and translate them into information controlling the synthesis and release of at least one family of pituitary hormones.

A particularly important laboratory experiment demonstrating neural control of the anterior pituitary was that of Everett and Sawyer (2) in the female rat. Injections of barbiturates in the late morning/early afternoon of pro-oestrous blocked ovulation in the early morning of oestrous with the animals ovulating 24 h later. This led to the concept of a 'critical period' during which a neuro-genic stimulus must lead to the release of gonadotrophic hormones to elicit ovulation.

The techniques of electrical stimulation and lesioning were fundamental to our understanding of the areas of the hypothalamus involved in controlling ovulation, beginning with relatively crude experiments in rabbits through to a more precise manipulation of discrete hypothalamic nuclei in rats.

The question then arose as to how the brain exerted its control. The most likely explanation was that there was a direct neural input to the anterior pituitary, but there was little evidence for such a pathway, leaving the possibility that there could be neuro-humoral mechanisms. This idea was formulated in the 1930s but fully developed by Geoffrey Harris, at that time working in Cambridge in the 1940s. The observation that there was a portal system of blood vessels draining the median eminence and running down the pituitary stalk specifically to supply the pituitary provided the link whereby factors released from axon terminals in the brain at local high concentrations could influence pituitary function (Fig. 1). The importance of this vascular link was demonstrated in experiments utilising a variety of elegant surgical techniques. Section of the pituitary stalk interrupted oestrous cycles in the rat. Over time, cycles returned, a phenomenon shown to be attributable to revascularisation by the portal vessels. Transplanting pituitary tissue into the sella turcica directly below the median eminence in hypophysectomised female rats resulted in revascularisation by portal vessels and restored full pituitary function. Transplants lateral to this site, whilst receiving a blood supply from non-portal vessels, failed to restore oestrous cycles. Transplantation of pituitary tissue away from the brain under the kidney capsule in hypophysectomised rats resulted in revascularisation by systemic vessels. Again there was no restoration of gonadal, thyroid or adrenal function, but the secretion of prolactin was unaffected. For the majority of pituitary hormones it appeared that hypothalamic control mechanisms were stimulatory, but for prolactin there appeared to be an inhibitory factor.

Nikitovitch-Winer and Everett (3) removed pituitary tissue from the sella turcica and transplanted it under the kidney capsule, with the expected result that gonadal activity was curtailed. They then retransplanted the same tissue below the median eminence with full restoration of oestrous cycles.

Over a period of more than 30 years up to the middle of the 1960s, the experimental evidence suggested that the brain produced factors that were stored in and released from the median eminence into the portal vessels to control anterior pituitary function. In the case of gonadotrophic hormone release, the results of electrical stimulation (Fig. 2), lesioning, and knife cuts interrupting neural pathways provided a consensus that a pathway from the medial preoptic area (MPOA) provided the major humoral input system to the pituitary. Several excellent reviews cover this early period of research (4–6).

With the establishment of the controlling influence of the brain over pituitary function, the challenge was now to isolate and characterise the active principles involved.

In order to demonstrate the presence of bioactive molecules controlling pituitary function in tissue extracts, there had to be assay systems capable of measuring the release of each pituitary trophic hormone. In the case of thyroid-stimulating hormone (TSH), the release of radioactive iodine-tagged thyroxin provided a sensitive bioassay for TSH and therefore for its releasing factor TRF.

Geoffrey Harris concentrated his attention on the control of luteinising hormone (LH) secretion and devised a method to assay release of this hormone in the reflex ovulating female rabbit. He



Fig. 1. The portal vessels running down the pituitary stalk (arrow) visualised after India ink injections.

placed an indwelling cannula directly into the anterior pituitary, thus bypassing the portal vessels. Infusion of hypothalamic extracts containing active factors would result in ovulation that would be easily detected by examination of the ovaries several hours later (Fig. 3).

Ascorbic acid is utilised as a co-factor in steroidogenesis, and a bioassay was developed to detect LH release in female rats with ovarian ascorbic acid depletion (OAAD) as the end-point measured.

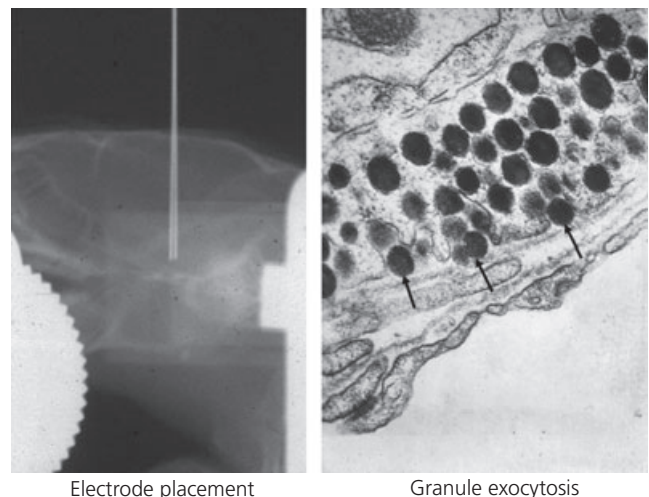


Fig. 2. An X-ray picture (left) of placement of a bipolar electrode into the medial preoptic area (MPOA) of a rat and the massive exocytosis of secretory granules (right; shown by arrows) from the median eminence following electrical stimulation.

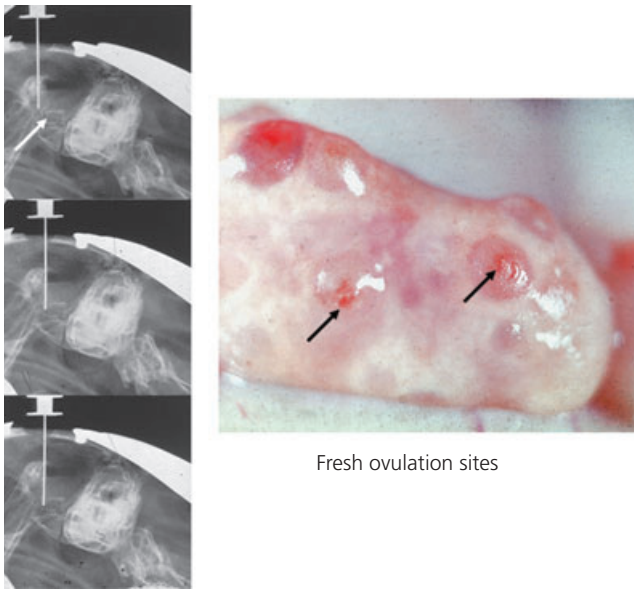


Fig. 3. The placement of a platinum cannula within the anterior pituitary gland of a female rabbit under X-ray guidance. The site of the sella turcica is arrowed. The right-hand image shows ovulation points in the ovary 24 h after infusion of a positive hypothalamic extract via the cannula.

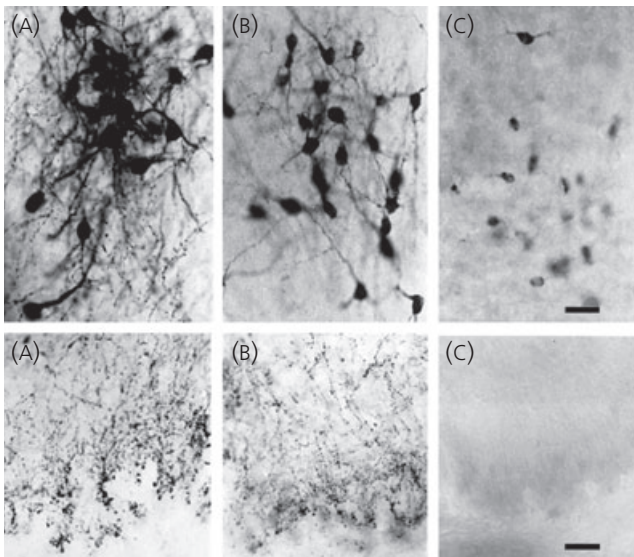


Fig. 4. Gonadotrophic hormone-releasing hormone (GnRH) staining of preoptic area (POA) neurones (upper panels) and median eminence (lower panels) in (A) an adult sexually developed starling (reprinted from (14)), (B) an immature bird and (C) a photorefractory animal.

This was the assay of choice for most laboratories attempting to isolate the luteinising hormone-releasing factor (LRF), as it was called in those days. This hypothalamic hormone is now referred to as the gonadotrophic hormone-releasing hormone (GnRH), and this abbreviation will be used in the rest of this article.

McCann *et al.* (7) were the first to report LH-releasing bioactivity from crude extracts of median eminence tissue using the OAAD

system, and Harris demonstrated similar activity in extracts of sheep hypothalami using the rabbit ovulation assay. When he moved to Oxford as Dr Lee's Professor of Anatomy, Harris also directed the MRC Neuroendocrinology Research Unit, part of which concentrated on the isolation of GnRH, initially with Peter Fawcett as the peptide chemist and later with Harry Gregory from ICI.

We are now considering the period between 1960 and 1970 and the methods for extraction, isolation, characterisation and assay available at that time. There were a variety of chromatographic techniques with perhaps the most important being the use of molecular sieving using Sephadex columns.

The bioassay systems described above were time consuming and labour and animal intensive, but in 1969, utilising millions of hypothalamic fragments, the tripeptide structure of TRH was established and gradually a picture built up indicating that the majority of hypothalamic factors controlling anterior pituitary function were peptides. There was one exception in that dopamine exercised a negative control over prolactin release.

There are several extensive reviews covering the techniques and personalities involved in the final elucidation of the structure of the hypothalamic releasing factors (8–11). The Nobel Lectures of Guillemin and Schally also make interesting reading.

Once the decapeptide structure of GnRH had been established by Schally in 1971 (12) and the molecule synthesised, then antibodies could be raised against it, allowing direct assay and also its visualisation in tissue sections.

One advantage of being in a department led by an international authority was that several visitors came to work with Harris. One of these, W. C. Worthington Jr, developed the technique of extracting blood from the pituitary portal vessels in rats. This was extended by George Fink, who demonstrated the presence of GnRH within portal blood extracts initially using the OAAD assay system. Another visitor was Fred Naftolin, who was instrumental in setting up radioimmunoassays in the department, and Fink successfully demonstrated that the surge of LH released into the general circulation in pro-oestrous followed a rise in portal blood GnRH (12). The use of portal bleeding techniques to investigate brain–pituitary interactions has been championed by Ian Clarke using the sheep as an animal model (13).

Cytological studies confirmed that the medial preoptic area was rich in GnRH cell bodies, with the axon terminals abutting the portal vessels in the median eminence. As might have been predicted, the MPOA and median eminence content of GnRH were shown to vary with reproductive status (Fig. 4) (14).

One of the most interesting observations on the GnRH neurone made possible using immunohistochemistry was the demonstration that most cells originated in the olfactory placode and migrated along with olfactory neurones into the central nervous system (CNS) during development (15).

Harris died in 1971, before the definitive structure of GnRH was established, but in 1977, by sheer serendipity, Bruce Cattanaach at Harwell had discovered a mouse mutant in which adults of both sexes exhibited extreme hypogonadism. He came to the Anatomy Department, by which time the laboratory of George Fink's group was set up for radioimmunoassay, and we demonstrated that these

mutants did not appear to make immunoreactive GnRH with a consequent reduction in pituitary gonadotrophic hormone content (16). The genetic cause of the mutation was a deletion in part of the GnRH gene, thus providing a natural knockout model (17). Injections of the decapeptide stimulated pituitary gonadotrophic hormone synthesis and secretion and gonadal growth. Peter Seeburg's group, utilising transgenic techniques, gave back the GnRH gene to these hypogonadal (hpg) mice with full activation of the pituitary and gonads (18). Dorothy Krieger suggested that the hpg mutants could provide a useful model to test the potential for neural grafts to restore physiological function. Placing normal neonatal MPOA tissue within the third ventricle of adult mutants resulted in full spermatogenesis in males and follicular development and pregnancy in females (19). Recently, Xandra Breakefield has injected viral vectors encoding the GnRH gene into the MPOA of hpg mice to reverse the effect of the mutation (20).

Whilst the structure of GnRH was reported in 1971, it was not until 1982 that the growth hormone-releasing hormone GHRH was purified. Interestingly, this factor was isolated from a pancreatic tumour in a patient exhibiting acromegaly without any evidence of pituitary cancer (21). This emphasises the point that research is based not only on laboratory experimentation but also upon clinical observation.

The laboratory experimental techniques available up until the advent of recombinant DNA-based methods were relatively crude; hypophysectomy removes a whole host of hormones and electrolytic lesioning is not cell specific. We can now ablate individual cell types utilising cell-specific promoters driving suicide genes, an approach used by Iain Robinson investigating the GHRH system (22). However, many neurones produce more than one transmitter/secretory product, so even specific cell ablation could be considered a fairly crude approach. Removal of a single gene product is possible using the cre-lox system, in which the cre-recombinase is activated by a cell-specific promoter to delete just one gene in transgenic mice. Silencing of specific genes has also been accomplished by using antisense RNA and in the last few years small interfering RNA (siRNA) molecules have been shown to block specific gene expression.

Immortalised cell lines for *in vitro* studies have been central to neuroendocrine research. By specifically targeting an oncogene to GnRH neurones, cell lines have been established for use in electrophysiological and biochemical studies (23). Modern methods have also enabled us to identify individual neuronal cell types in brain slices by utilising specific promoters to drive a molecule such as green fluorescent protein (GFP) to visualise the cells not only for electrical stimulation and recording but also for individual cell PCR analysis (24, 25).

An important aspect in dissecting out control mechanisms within the CNS is to determine which pathways from other parts of the brain impinge on a particular cell type. Close apposition of axon terminals observed with light microscopy/confocal microscopy, whilst suggestive of input from one neurone to another, does not provide conclusive evidence. Viruses can infect and replicate in one cell, releasing millions of particles when the cell dies. These can be taken up by nerve terminals abutting onto the infected cell body,

thus infecting first-order input neurones which will themselves die and release virus to be taken up by second- and third-order neurones in a pathway. This method has been adapted to trace potential input pathways to the GnRH cell by producing transgenic mice in which the cre-recombinase gene has been targeted to GnRH neurones. In such mice, if a non-replicating pseudorabies virus in which the cre-recombinase can excise a stop codon and also express a GFP is injected into the MPOA, then only transfected GnRH neurones will allow replication of the virus and the production of GFP, providing a means to follow the uptake and release of virus from one group of neurones to another in tract tracing experiments. Alan Herbison's group has used this technique to identify brain stem afferents to GnRH neurones (26).

Over the last few years, a whole new layer of information has been added to our understanding of brain-pituitary-gonadal interrelationships. This has been made possible by the enormous advances in recombinant DNA technology, protein isolation and structural analysis. Added to this is the serendipitous nature of research where one branch of study is found to impact on unexpected and unrelated fields.

Over a decade ago, a group of cancer biologists were attempting to identify a factor that inhibited metastases of melanoma cells. By comparing the RNA profile of a cell line that was metastatic with a non-metastatic line using differential display, they identified a messenger RNA predicted to encode a 164-amino acid (aa) protein, KISS-1 (27). A few years later a G-protein coupled receptor (GPR54) for this peptide was identified (28). How was this accomplished?

The tissue distribution of GPR54 mRNA was determined using RT-PCR and the gene was shown to be highly expressed in human placental tissue. In the expectation that the placenta may also be rich in the ligand for the receptor, homogenised extracts were loaded on to high-performance liquid chromatography (HPLC) columns and 50-ml fractions collected for bioassay. The importance of bioassays in the isolation of active peptides has already been mentioned in the search for hypothalamic releasing factors. In the case of GPR54, Chinese hamster ovary cells (CHO-K1), which do not express the receptor, were transfected *in vitro* with plasmids encoding the receptor. These cells had already been modified to respond to receptor signalling by activating a luminescent protein, providing a rapid and sensitive assay for any peptides in the extract that would activate the receptor. After initial screening and further HPLC isolation, active fractions were analysed by mass spectrometry to yield two short overlapping peptides of 13 and 14 aa, and a longer related 54-aa protein. The structure of these three peptides was fed into the database and all were predicted to be derived from the kisspeptin protein. All of this research took place over a relatively short period of time and used small amounts of starting material for isolation. The design of the cell line for the assay and the use of HPLC for isolation and mass spectrometry for structural analysis, coupled to proteomic and genomic databases, were the keys. Compare this to the enormous numbers of sheep and pig hypothalami used by Schally and Guillemin and their initial isolation techniques, assay systems and methods of peptide analysis. What took years in the 1960s and 1970s would nowadays occupy only a matter of months.

The protein and receptor were obviously going to be of value in investigating the invasiveness of tumour and placental cells, but what has this got to do with hypothalamic control of the pituitary?

Again, the value of clinical observation was important. In 2003, human geneticists, using linkage analysis and candidate gene screening, identified mutations in GPR54 associated with familial hypogonadism (29). Collaborators knocked out this gene in mice, as did a second group, producing mutant mice in which postnatal gonadal development was disrupted, with atrophic seminal vesicles and a failure of full spermatogenesis in males and a thread-like uterus and ovaries lacking large follicles in females. The pituitary content of LH and follicle-stimulating hormone (FSH) was lower than in normal mice, but significantly higher than in hpg mutants.

GPR54 mRNA is found in GnRH neurones within the hypothalamus of normal mice, suggesting that kisspeptin could directly control the activity of these cells. GPR54KO mice possess a normal complement of GnRH within the hypothalamus and median eminence. It would appear that, although the synthesis of the decapeptide is not obviously compromised, control of its release is abnormal. Recently, kisspeptin knockout mice have been generated and their phenotype closely mirrors that of the GPR54 mutants (30, 31).

There are three major sites of kisspeptin production in the hypothalamus in rodents: the anteroventricular periventricular area (AVPV), the periventricular nucleus and the arcuate nucleus. The AVPV group of kisspeptin cells are likely to represent the site at which oestrogen may play a positive feedback role in the surge of LH associated with ovulation, whilst the arcuate nucleus may be involved in a more tonic input.

The host of modern techniques available to the neuroendocrinologist means that we should soon be able to identify pathways influencing kisspeptin neurones, probe the electrophysiological properties of these cells and perhaps identify potential interplay between populations of cells transducing the effects of nutrition, day length, stress, etc. upon reproduction. The identification of factors involved in these processes will also allow chemists to synthesise agonist and antagonist compounds, just as the earlier work on the releasing factors led to significant drug development. We must also not forget the unknown input that serendipity will play in future discoveries.

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