Corresponding author mail id: smoenter@umich.edu

Gonadotropin-releasing hormone (GnRH) measurements in pituitary portal blood: a history

Suzanne M. Moenter and Neil P. Evans

[1] Departments of Molecular & Integrative Physiology, Internal Medicine, Obstetrics & Gynecology, and the Reproductive Sciences Program[4], University of Michigan, Ann Arbor, MI, 48109, USA

[2] Institute of Biodiversity Animal Health and Comparative Medicine, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, G128QQ , UK

Running title: GnRH measurements in pituitary portal blood

Disclosures: The authors have nothing to disclose

Funding: The authors acknowledge funding from the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development grants R37HD034860, R01HD041469 and R01HD104345 to SMM.

Data Availability Statement: Data sharing not applicable – no new data generated

Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; E, estradiol

Summary

Much about the neuroendocrine control of reproduction is inferred from changes in the episodic release of luteinizing hormone (LH), as measured in samples of peripheral blood. This, however, assumes that LH precisely mirrors gonadotropin-releasing hormone (GnRH) release from the hypothalamus. As GnRH is not measurable in peripheral blood, characterization of the relationship between these two hormones required the simultaneous measurement of GnRH and LH in pituitary portal and peripheral blood, respectively. Here, we review the history of why and how portal blood collection was developed, the aspects of the true output of the central component of the hypothalamo-pituitary-gonadal axis that this methodology helped clarify, and conditions under which the pituitary fails to serve as an adequate bioassay for the release pattern of GnRH.

Keywords

Transsphenoidal, luteinizing hormone, science history

Introduction

A role for the central nervous system in the control of reproduction was initially provided over eighty years ago, by Francis Marshall and Ernest Verney (1), and Geoffrey Harris (2) who published work in rabbits that demonstrated ovulation could be induced by stimulation of the brain. Earlier work had demonstrated a role for the pituitary gland in gonadal growth and estrous cyclicity through ablation and replacement studies (3-5). The work of Marshall, Verney and Harris opened an additional area for investigation, beyond the pituitary, for the control of reproduction. Specifically, the central nervous system appeared to have a critical role

Not long before the work of Marshall, Verney and Harris, Gregor Popa and Una Fielding had identified the hypothalamic-hypophyseal portal vascular system, which connects the median eminence of the hypothalamus to the anterior portion of the pituitary gland (6). While the direction of blood flow through this capillary system was initially debated, with Popa and Fielding proposing that blood flowed from the pituitary towards the brain, George Wislocki and Lester King soon convincingly established that the direction of flow was from the brain towards the pituitary (7). These hypothalamic-hypophyseal or pituitary portal capillaries, as they will be referred to from this point,

provided a vascular route of communication between the hypothalamus and the anterior pituitary gland that could help explain the accumulating findings of central regulation of pituitary function.

Harris was a leading advocate of the neuroendocrine hypothesis for the control of anterior pituitary and reproductive function, concluding in a review (8) that “it seems possible that nervous stimuli might cause the liberation of some substance into the capillary sinusoids of the median eminence, this substance then being transported via the hypophysial portal vessels to excite or inhibit the pars distalis”. The idea that the central nervous system would control something as lowly as hormone release was not popular among some of the leading neurobiologists of the day (9, 10), despite being supported by some key observations. For example, Otto Loewi’s classic studies in frog hearts provided strong evidence for “humoral” transmission in the peripheral nervous system. While it was known that vagal stimulation could slow heart rate in an *ex vivo* heart preparation, Loewi showed that the fluid that had bathed such a preparation could slow the rate of a similarly-prepared heart in the absence of vagal stimulation (11). This observation suggested a humoral mediator, which Loewi termed “Vagusstoff”. Vagusstoff had activity similar to that of acetylcholine, which Henry Dale, Loewi’s co-recipient of the 1936 Nobel Prize in Physiology and Medicine, later demonstrated was made by the body (12). Despite such evidence, some, led primarily by the future Nobel laureate (1963) John Eccles, remained convinced that neural transmission, particularly within the central nervous system, was too fast to be chemical and thus must be electrical. It was Eccles own work in the middle of the last century that provided the definitive evidence that electrical signals on their own could not reproduce the changes in membrane polarization that were observed as intracellular recording methods became available (13, 14). While the neural factors postulated by Harris were disputed by some, others joined a relentless quest to identify them. This quest was advanced by Andrew Schally and Roger Guillemin, who sequenced several secreted neural factors including GnRH (15, 16) and in so doing promoted them from factors to hormones. Identification of these factors accelerated ongoing efforts to find evidence for these substances in pituitary portal blood.

Earliest measurements of portal blood

The first assessments of the functional contents of portal blood were made by collecting effluent from the severed pituitary stalk, typically of rats. Initial work investigated non-reproductive functions (e.g., (17)), but in 1967, the efficacy of pituitary stalk blood vs peripheral blood obtained from the same rat was tested in the ovarian ascorbic acid depletion assay, an early bioassay for LH (18). Stalk blood

was more effective than peripheral blood in depleting ascorbic acid; this suggested the releasing factor contained in stalk blood produced an increase in bioactive LH to a level greater than that in the peripheral blood from the same donor (19). Importantly, the possibility that this activity was attributable to contamination of the stalk blood with pituitary hormones from the surgical field, specifically LH itself, was mitigated because a similar result was obtained on samples from hypophysectomized rats. Subsequent work confirmed these results using the alternative LH bioassay of ovulation induction in rabbits (20). GnRH, as measured by radioimmunoassay, was next shown to be elevated in portal blood at the typical time of the proestrous LH surge in rats (21), and in rabbits during the cupric acid-induced LH surge (22) In addition, studies using sequential samples of stalk blood from rhesus monkeys, demonstrated that GnRH concentrations fluctuated in portal blood and that the fluctuations were more prominent in ovariectomized monkeys, suggesting the hypothalamus could drive pulsatile pituitary LH release (23).

These studies were without doubt innovative and provided important proof of principle that GnRH-like activity was detectable in stalk blood but rarely in peripheral samples. They argued for a central source of GnRH but were hampered by three primary caveats. First, the surgical approaches (transsphenoidal or transorbital) necessitated that sample collection be done under anesthesia, a substantial drawback for the study of central neural function. Second, severing the stalk compromised the ability to measure simultaneously the postulated releasing hormone, GnRH and the pituitary tropic hormone, LH. Third, the sampling window was typically brief (under 2 h), due to the sample collection protocol and small body size of the species used, precluding study of the patterned release that is the hallmark of this system (24).

The latter two caveats were overcome in a study in sheep, soon to be the dominant species for this research, by Alain Caraty of the Institut National de la Recherche Agronimique, Station de Physiologie de la Reproduction in Nouzilly, France. Caraty's approach used x-ray-identified landmarks to guide the surgical implantation of a tube containing concentric cannulae between the hemispheres of the brain, so that the tip of the cannula was near the anterior face of the pituitary. While the animals were still under anesthesia, a stylet was used to lesion the portal vessels and a solution containing heparin (an anticoagulant) and bacitracin (a protease inhibitor) was perfused through the outer cannula and collected via the inner cannula using a peristaltic pump. Using this method, Caraty was able to demonstrate a distinctly pulsatile pattern of GnRH secretion, but the

coincidence with LH pulses was not as evident, perhaps attributable to the approach or anesthesia(25).

A series of fateful meetings and international collaborations: as told by Fred Karsch to Sue Moenter in the late 1980s

The next major step forward with regard to the measurement of GnRH in pituitary portal blood was triggered in February 1980, in Leura Australia, at a satellite symposium associated with the Sixth International Congress of Endocrinology. The topic of the symposium was *Reproductive Endocrinology of Domestic Ruminants*, and it brought together what would be two of three key players in portal blood collection: Iain Clarke, then at Prince Henry's Hospital in Melbourne Australia, and Fred Karsch from the University of Michigan. Discussion among the meeting participants either after one of the talks or later in the pub, turned to the relationship between hypothalamic releasing hormones and their anterior pituitary counterparts. Fred Karsch recalled Iain Clarke stating that what was necessary was simultaneous collection of samples of pituitary portal and peripheral blood from conscious normally-behaving animals. While eminently logical, this was apparently met with skepticism by the conference participants as to its practicality.

Undeterred, and perhaps even inspired, Clarke returned to Melbourne and looked up neurosurgeons in the phone book, searching for someone to help develop such an approach. James Cummins proved a willing partner. In 1982, their pioneering work led the first publication to measure simultaneously GnRH in the pituitary portal and LH in the peripheral blood of conscious sheep(26). They pioneered a surgical transsphenoidal approach to access the pituitary, create an artificial sinus in the bone in front of the pituitary and implant two needles near the frontal face of the pituitary, securing these to the nasal bones with dental acrylic. Two days after surgery, the conscious sheep were heparinized and a stylet placed through the upper needle was used to cut some, but importantly not all, of the pituitary portal capillaries running down the anterior face of the pituitary gland. Blood that pooled in the artificial sinus was collected via the lower needle using a vacuum pump, the upper needle serving as an air vent. At the same time jugular blood was collected via an indwelling cannula. Their data revealed that in ovariectomized ewes, each LH pulse in the peripheral blood had a corresponding GnRH pulse measured in pituitary portal blood, providing solid confirmation for neuroendocrine control. This study also raised questions, however, as not every increase in GnRH in portal blood had a corresponding LH pulse, leading to postulates about the role

of silent GnRH pulses in pituitary function. Cummings and Clarke themselves recognized some caveats of this method, specifically the potential contamination of portal blood samples with cerebrospinal fluid, detected by the reduction in hematocrit of portal compared to peripheral blood samples. Further, the short recovery time post-surgery and open artificial sinus made it possible that peripheral blood from the surgical field might also accumulate in the collection sinus in the heparinized sheep, thus diluting portal samples and precluding accurate measurement of GnRH.

In 1984, Fred Karsch did a sabbatical in Iain Clarke's laboratory during which he learned the surgical approach for collecting pituitary portal blood developed by Clarke and Cummins while performing collaborative studies on steroid regulation of GnRH and LH release (27). Following this, the Karsch family traveled back to the USA, via Europe, visiting Alain Caraty's group in Nouzilly in April 1985. This was the first time that Fred Karsch met Alain Caraty, who was also working on a portal blood collection method. Fred shared pointers he had learned during his sabbatical with Iain Clarke. In September 1987, Karsch returned to Nouzilly for the Colloquium on Neuroendocrine Mechanisms and Light Control of Reproduction in Domestic Mammals, and was brought up to date on how Caraty, with his surgical collaborator Alain Locatelli, had altered the portal blood sampling method.

The Caraty and Locatelli approach included several modifications, which would increase the rigor and reproducibility of the measurements (Figure 1). First, the surgical field was smaller, resulting in less disruption of tissue *en route* to the pituitary, although this was still considerable in the nasal turbinate region. Second, rather than creating an artificial sinus for blood collection that was contiguous with the surgical field, this approach used a device with a collection reservoir that effectively isolated portal blood from other fluids. This device, the 'gadget' as it was called in Nouzilly or 'gizmo', as it became called in the USA, was constructed as follows. Two blunt three-inch needles (one 12 gauge and one 14 gauge) were bonded together with dental acrylic. Then, a small sleeve made from a 1.5 ml microcentrifuge tube was secured over the blunt ends of the needles, forming a plastic cup at the end of the device that served as a small collection reservoir. To place the gadget, a triangle of the frontal and nasal bone between and below the supraorbital foramen was excised, then sections of the nasal turbinates were removed, and a tunnel was drilled through the cribriform plate of the ethmoid bone, ventrocaudally under the olfactory bulbs and optic chiasm. Upon reaching the face of the sphenoid bone, a hole was carefully created in front of the pituitary, and the dura covering the pituitary cut away. The gadget was placed in the tunnel so that the plastic cup rested on the bone in front of the pituitary, over the hole. A third modification that increased the consistency of the

measurements was to fill the entire surgical field with dental acrylic, rather than just securing the collection needles to the nasal bones. This, in combination with the plastic cup, reduced the possibility of contamination of pituitary portal blood samples with peripheral blood from the surgical site. The cup also essentially precluded entry of cerebrospinal fluid into the collection area, as confirmed by the similar hematocrits of pituitary portal and peripheral blood throughout the sampling period. Fourth, filling the surgical field with dental acrylic also increased stability of the collection device, allowing for a longer post-surgery recovery period before heparinization and blood collection, typically 1-2 weeks, increasing healing time and further reducing the likelihood of peripheral blood contamination of pituitary portal blood samples. Like the original approach of Clarke and Cummings, sheep were heparinized on the day of collection, and a small portion of pituitary portal vessels were lesioned by a stylet placed through one of the needles. Blood was withdrawn using a peristaltic pump. [Full details are provided in(28)].

The hormone data obtained with this method were remarkably clear. The first publication from the Caraty group was on the effects of the opiate receptor antagonist naloxone, which had been shown to increase LH release in rams(29). To test if this was at the central and/or pituitary level, simultaneous samples of pituitary portal and jugular blood were made from four conscious short-term castrate rams(30). They found that in short-term castrated rams before treatment with naloxone, clear and completely coincident pulses of GnRH and LH were observed. A single injection of naloxone increased the amplitude of both GnRH and LH release, but coincident pulses were still observed. Multiple naloxone injections had a further effect to increase the frequency of pulsatile GnRH release. During this high frequency GnRH pulse barrage, a sustained elevation in LH was observed but pulses became obscured. This is likely due to a combination of biologic and technical factors. Biologically, readily releasable stores of LH may have been diminished leading to less distinct increases in LH in response to each GnRH pulse. Further, the GnRH frequency was about one pulse per 20 min, perhaps providing inadequate time for LH levels in the peripheral circulation to decay by the required metrics for pulse detection. Technically, the frequency of LH sampling may not have been adequate to observe clear pulses at this higher frequency of GnRH input. Sampling the portal blood would also diminish the amount of GnRH available to bind to pituitary receptors. Of note in this regard, LH pulses were clearly visible during the control period, suggesting it was the increase in GnRH pulse frequency that primarily led to the elevated but not strictly pulsatile LH signal.

A similar phenomenon was observed in the next paper from the Caraty group, in which the effect of time after castration upon GnRH and LH was examined in male sheep(31). In gonad-intact rams and in wethers castrated 1-15 days before sampling, clear and completely coincident pulses of GnRH and LH were observed. GnRH pulse interval was longer in intact rams than in these short-term castrate males. A further reduction in GnRH pulse interval was observed in long-term (1-5 months) castrate males. In these animals, however the clearly episodic high frequency of GnRH release was again not reflected in distinct LH pulses. Notably, this study included a period of jugular sampling before lesioning the pituitary portal vessels. From these samples, it could be seen that LH pulses were often unclear even before portal sampling began in the long-term castrate males, indicating loss of some portal blood to collection was not the cause of LH irregularity. In combination with the above study, these findings suggest the GnRH pulse generator can operate in a distinctly episodic manner at frequencies that are too high for pituitary output as measured by LH release to clearly reflect.

GnRH release during the female reproductive cycle and the estradiol-induced LH surge

One of the primary questions of the day was what happened to GnRH release at the time of the LH surge. There were two main schools of thought. One was based on data from Ernst Knobil's group that monkeys with lesioned hypothalami exhibited menstrual cycle-like changes in gonadotropins and ovarian steroids, including LH surges, when GnRH was replaced at one pulse per hour(32). This suggested that while GnRH was required, the pattern did not need to change for a surge to occur. The other thought was that changes in GnRH would be needed to drive the LH surge. This stemmed from observations in sheep that showed a large increase in GnRH administration was required to induce an LH surge(33, 34), and early reports of GnRH/GnRH-activity increasing in portal-only preparations during LH surges in rats and rabbits(21, 22).

Prior studies in sheep had not provided a clear consistent answer to this question. The Clarke lab had published a study examining the natural estrous cycle, defining three patterns at the time of the LH surge in sheep: a large signal pulse of GnRH that occurred at the onset of the LH surge, a persistent increase in GnRH and no change in GnRH(35). Both the Clarke and Caraty laboratories had examined the surge induced by injection of pharmacologic levels of estradiol benzoate or estradiol, respectively, to ovariectomized ewes(36, 37). Caraty observed an initial negative feedback response for both GnRH and LH release in response to steroid injection, followed by positive

feedback induction of clear sustained surges of both GnRH and LH, with apparent loss of episodic release. Clarke saw no shift in GnRH pulse frequency during estrogen negative feedback compared to ovariectomized controls but observed an increase in GnRH pulse frequency during positive feedback. These studies all suggested a change in GnRH might occur but lacked consensus of approach and results. Did the inconsistencies in the natural cycle study reflect true biologic variation or technical challenges? Was the consistency of the Caraty study attributable to the high dose and route of administration of estradiol? What did a lack of negative feedback imply in the study by Clarke, et al.? How does prior removal of progesterone (more recently present in the cycling sheep) alter the response to increased estradiol?

A series of studies were undertaken to address these questions. The first published used an established model of the follicular phase of the ewe(38). Sheep were ovariectomized and fitted with a portal blood collection gadget in the middle of the luteal phase; at this time Silastic implants producing physiologic levels of progesterone and estradiol were placed. Approximately one week later, at what would have been the time of luteolysis in ovary-intact ewes, the progesterone implants were removed to simulate this process, and the sheep divided into two groups. In one, the luteal phase estradiol (E) implant was removed (no E). In the other, additional estradiol implants were inserted 16h after progesterone removal, raising concentrations to those seen in the mid follicular phase (E rise); this treatment reliably induces a surge ~21-24 h after the E rise. This artificial follicular phase model helped time the portal sampling to coincide with the expected LH surge in the E rise group(39). In the no E group, GnRH and LH were strictly episodic with coincident pulses. In marked contrast, ewes in the E rise group had suppressed GnRH and LH levels at the start of sampling but all of these ewes exhibited a robust GnRH surge. This surge began at the same time as the LH surge, but extended several hours longer than the LH surge, which was of normal duration. Repeating this model with the addition of an artificial luteal phase, during the anestrous season produced similar results (40). These data clearly showed consistency of effect of a physiologic level of estradiol, and that removal of progesterone alone was insufficient to induce a surge mode of GnRH release.

To confirm the findings of the artificial follicular phase model were representative of the natural cycle, a collaborative study was conducted between the laboratories of Karsch (Suffolk ewes) and Caraty (Ile de France ewes) (41). In this study, ewes were again fitted with portal blood collection gadgets in the luteal phase. Some ewes were sampled later in that same luteal phase (day 9-13 after ovulation).

Others were to be sampled during the subsequent follicular phase; these ewes received Silastic implants producing luteal phase levels of progesterone at the time of portal surgery. These implants transiently elevated progesterone levels until the end of the luteal phase, when progesterone falls with regression of the corpus luteum. The onset of the next natural follicular phase was timed by removal of the implants two days after luteolysis was anticipated. GnRH and LH were examined during the natural luteal phase (no progesterone implants) or timed natural follicular phase. During the luteal phase, GnRH and LH pulses were low frequency (1-2 per 5 h) and coincident. The frequency of both GnRH and LH pulses increased in the early follicular phase to about one pulse per hour, and pulses remained clearly coincident. As the follicular phase progressed, the frequency of GnRH pulses increased further. As had been observed in the long-term castrate and naloxone-treated males, the LH pulse pattern deteriorated at these higher GnRH pulse frequencies, making LH-pulse detection difficult. Twelve ewes were sampled during the preovulatory LH surge. Eleven of these had a clear increase in GnRH during the LH surge. In the one ewe not exhibiting a GnRH surge, autopsy revealed that the stylet used to lesion the vessels would have impinged on the sphenoid bone rather than the portal vessels, an exception that proved the rule. In animals sampled past the time of the LH surge, the GnRH surge was again extended. This observation in the natural follicular phase was important because it demonstrated that the prolonged GnRH surge in the artificial follicular phase model was not an artifact of continued exposure to high physiologic estradiol levels maintained by the implants (estradiol typically begins declining at the start of the LH surge). Together these studies demonstrated that at least in sheep, a GnRH surge is consistently produced at the time of both the preovulatory and estradiol-induced LH surges.

What is the pattern of GnRH release during the surge? The GnRH surges observed above appeared to be a continuous elevation, a striking contrast to the clearly episodic pattern of GnRH release that had been described at other times of the cycle in ovariectomized and ovary-intact ewes (26, 34, 36, 38, 40) and in rams (42). To assess the changes in the pattern of GnRH secreted during the surge, pituitary portal blood samples were collected form short-term ovariectomized ewes and ewes in the artificial follicular phase model described above with different sampling frequencies (30-s to 2-min intervals). The higher-frequency sample collection was important to exclude the possibility that the 10-min sampling interval used previously was not sufficiently frequent to detect distinct pulses, if the frequency of GnRH release was very high. GnRH pulses were easily detected at a sampling interval as short as 30 s in ovariectomized ewes; pulses were clear and abrupt increases that were sustained

for several minutes before rapidly returning to the interpulse level, which was low to undetectable (43). But even this high sample frequency failed to identify discrete pulses during the GnRH surge (44), suggesting the surge is a different mode of release.

Effects of estradiol on GnRH dynamics

The data up to this point indicated that, in the female sheep under the influence of follicular phase concentrations of estradiol, the patterns of GnRH release changes from being pulsatile, i.e., discrete periods of GnRH release, to a surge mode during which GnRH concentrations remain elevated for many hours. In order to investigate these estradiol-induced changes in GnRH secretion in more detail, a study was conducted using a modification of the artificial follicular phase model in which ewes received: no E (luteal phase E implant removed), basal E , and increasing E, in which additional estradiol implants were provided every 6-7 h to reach the levels in the E rise group (45). In samples collected every 10 min, it could clearly be seen that estradiol reduced GnRH pulse amplitude and increased GnRH pulse frequency in a dose-dependent manner across the 'artificial follicular phase' and prior to the GnRH surge. This is similar to what had been observed through the natural follicular phase when estradiol synthesis by the ovary was increasing.

While the above study clarified changes in pulsatile GnRH release during negative feedback it did not address whether estradiol induced the LH surge through changes in pulsatile GnRH secretion or a more profound change in which there is at least some component of continuous GnRH release, perhaps arising from a separate population of GnRH neurons. The question of whether different populations of GnRH neurons produced the surge vs pulsatile modes of release had been raised in classic knife-cut studies, largely in rodents, which suggested that the preoptic neurons may be more important for the surge, whereas more caudal neurons in the medial basal hypothalamus were responsible for pulse generation(46-48). This postulate was supported by work in sheep using cFos as a reporter of neural activity; exposure of ewes to novel rams is known to cause an abrupt increase LH pulse frequency and this treatment increased cFos in more caudal cells within the medial basal hypothalamus (49). During the preovulatory surge, however, it was primarily preoptic GnRH neurons that coexpressed cFos in rats, whereas GnRH neurons throughout the continuum expressed cFos in sheep (50-52). More recent work has suggested this dichotomy may be attributable to different properties of and inputs to GnRH neurons that depend upon the region (e.g., soma vs terminals) (53).

To address this, the changing patterns of GnRH and LH release at the start of the estradiol induced surge were characterized by means of 1 and 10 minute samples, respectively, over an eleven hour period spanning the expected start of the surge and in shorter windows in ovary intact ewes in the natural follicular phase(54). The results demonstrated highly consistent, characteristic changes in GnRH secretion across all of the ewes studied. Specifically, GnRH secretion was initially discretely pulsatile, but as the surge approached GnRH became detectable between pulses. This was followed by a period during which there was augmentation of both pulsatile and 'baseline' GnRH secretion, after which GnRH remained elevated and variable but during which time discrete pulses of GnRH could not be identified. The results, therefore, favored actions of estradiol to result in not only quantitative changes in pulsatile GnRH release but to also to alter the mode of GnRH secretion(55). To determine that the GnRH released as a result of these changes in the pattern of GnRH secretion is equally bioactive, despite termination of the LH surge many hours before the end of the GnRH surge, biological activity of the GnRH surge was investigated by timed blockade of GnRH receptors with the reversible GnRH antagonist Nal-Glu, analysis of GnRH immunoreactivity across the surge, and with an ovine pituitary bioassay (62). The results of all assays indicated that the GnRH observed in pituitary portal blood was equally bioactive across the surge, indicating that the LH surge does not end because of a change in GnRH bioactivity.

The above studies clearly demonstrated that the GnRH surge depends on estradiol, with a consistent latency of about 21 hours from estradiol rise to surge onset in sheep. The actions of estradiol to trigger the GnRH and LH surge are likely act via estrogen receptor alpha (56-58). As this receptor does not appear to be expressed in GnRH neurons it suggests that estradiol-sensitive afferents are required to process the surge signal. The portal blood collection methodology was used to investigate if the entire latent period of estradiol exposure was required to generate a surge, or if estradiol might trigger changes in steroid-receptive systems that are activated to drive the GnRH surge that become irreversible (59). This study documented that the GnRH surge did not require estradiol to be elevated at the time of the surge for expression of a GnRH surge of normal amplitude that extended beyond the LH surge, but that the duration of the GnRH surge duration was longer when the E rise was maintained. Further, shortening of the estradiol signal suggested that a duration of between 7 and 14 h of estradiol exposure, in advance of surge onset, was all that was necessary to induce a consistent GnRH/LH surge. Together these findings are consistent with the existence of a critical period for estradiol-dependent activation of neural systems to drive the GnRH surge, and

are in agreement with classic studies of barbiturate blockade of ovulation in rats(60, 61), and the persistence of alterations in GnRH neuron activity in mice induced by estradiol feedback after preparation of brain slices for recording these cells(62, 63).

What else has portal blood sampling told us about the GnRH neurosecretory system?

Another central action of GnRH that has been postulated is whether it has effects upon its own release(64). The lack of effect of Nal-Glu on the GnRH surge above suggests this is unlikely. Prior work had also shown no effect of either GnRH receptor agonists (0.5 mg D-Trp6-GnRH im) or antagonist (5mg Nal-Glu im) upon release of the endogenous decapeptide in short-term castrate rams(65). A similar lack of an effect was observed in a study that combined intracerebroventricular cannulation and pituitary portal blood collection to ascertain if infusion of GnRH into the lateral ventricle supported an ultrashort feedback loop role for GnRH released into the cerebrospinal fluid on GnRH secretion(66). In contrast, a study in female sheep found that lower doses of Nal-Glu (10mg/kg iv) increased GnRH pulse frequency in a subset of ovariectomized ewes(67). Interestingly, this effect was more consistent and pronounced in luteal phase ewes and ovariectomized ewes treated with estradiol and progesterone to mimic the luteal phase. Together, these observations suggest that the steroid milieu and initial GnRH pulse frequency may both determine if GnRH can affect its own release.

Sampling of pituitary portal blood has been used to answer other questions about the neuroendocrine systems. Progesterone blocks the LH surge in sheep by blocking the GnRH surge; these effects are mediated by the classical progesterone receptor(68, 69). Masculinization of the sexually-indifferent fetal hypothalamus by testosterone abolishes the GnRH surge, demonstrating this treatment blocks the positive feedback effects of estradiol at the level of the hypothalamus (70). Changes in GnRH release also underlie seasonal changes in LH sensitivity to estradiol feedback between the breeding season and anestrus (71). Thyroidectomy blocks the seasonal decline in GnRH pulse frequency between the breeding and anestrous seasons (72). Thyroidectomy increases thyrotropin releasing hormone levels in portal blood but this hormone is not pulsatile (73). Opioid peptides alter the shape of GnRH pulses and GnRH release both in the presence and absence of estradiol, demonstrating opioids have effects beyond mediating steroid feedback (74). The powerful GnRH secretagogue kisspeptin is identifiable in pituitary portal blood, but levels did not change during the LH surge perhaps indicating neuromodulatory rather than any neuroendocrine effects of

kisspeptin are dominant at that time (75). In this regard, pulsatile administration of kisspeptin 10 generates GnRH and LH pulses, whereas a sustained kisspeptin 10 infusion leads to a sustained GnRH elevation in portal blood with no evidence of GnRH pulses (76). Finally, follicle-stimulating hormone was shown to have both an episodic and constitutive release when measured in portal blood (77). None of these observations would have been possible if only jugular blood was measured. Perhaps the area most investigated after changes in GnRH release with gonadal steroid feedback is the interactions of the stress and reproductive neuroendocrine axes; these studies are reviewed by McCosh et al., in this volume.

Summary

The ability to sample simultaneously pituitary portal blood to measure releasing hormones and peripheral blood to monitor pituitary output has markedly increased our understanding of reproductive neuroendocrine function. While LH pulses remain a good bioassay for GnRH in many conditions, the studies described above indicate that when GnRH release is high frequency, such as during the late follicular phase, after long-term steroid removal or some drug treatments, the LH signal may become less clear. This can lead to the misinterpretation that these conditions are associated with reduced GnRH release, a possibility that can be convincingly dismissed by sampling portal blood. Portal sampling also revealed a markedly different duration of estradiol positive feedback effects upon GnRH release than at the pituitary and have opened further questions regarding central GnRH action.

Reproductive neuroendocrinology has continued to evolve since portal blood collection was state-of-the-art. Large animal models are not as readily available now, and the genetic tools available in rodents, particularly mice, have opened exciting new venues and methodologies to elucidate the central circuits controlling fertility. The data reviewed here make a good argument for measuring some aspect of central function, whether it be portal blood, neural activity or changes in intracellular calcium, to confirm if changes in LH are paralleling central changes in reproductive neuroendocrine function. This is particularly true with modern neurobiologic tools that have the ability to push the GnRH system to the high functioning states when LH does not serve as a good readout of central activity. Even with sensitive assays for LH release in mice(78), it is worth bearing in mind that LH can go down when GnRH activity is very high, a mismatch that can potentially lead to profoundly different interpretations if only the peripheral system is assessed.

Literature Cited

1. Marshall FHA, Verney EB. The occurence of ovulation and pseudo-pregnancy in the rabbit as a result of central nervous stimulation. *J Physiol*. 1936; **86**(3): 327-36.

2. Harris GW. The induction of ovulation in the rabbit by electrical stimulation of the hypothalamo-hypophysial mechanism. . *Proceedings of the Royal Society of London Series B, Biological sciences*. 1937; **122**(828): 374-94.

3. Smith PE. The disabilities caused by hypophysectomy and their repair: The tuberal (hypothalamic) syndrome in the rat. *JAMA*. 1927; **88**(3): 158-61.

4. Smith PE. Ablation and transplantation of the hypophyses in the rat. *Anat Rec*. 1926; **32**(3): 221.

5. Evans HM, Long JA. The effect of the anterior lobe of the hypophysis administered intraperitoneally upon growth and maturity and oestrous cycles of the rat. . *Anat Rec*. 1921; **21**(1): 62-3.

6. Popa G. A Portal Circulation from the Pituitary to the Hypothalamic Region. *J Anat*. 1930; **65**(Pt 1): 88-91.

7. Wislocki GB, King LS. The permeability of the hypophysis and hypothalamus to vital dyes, with a study of the hypophyseal vascular supply. *Am J Anat*. 1936; **58**(2): 421-72.

8. Harris GW. Neural control of the pituitary gland. *Physiological reviews*. 1948; **28**(2): 139-79.

9. Raisman G. An urge to examplin the incoprehensible: Geoffrey Harris and the discovery of the neural control of the pituitary gland. *Ann Rev Neurosci*. 1997; **20**(1): 533-66.

10. Wade N. *The Nobel Duel* Garden City, NY: Anchor Press/Doubleday, 1981.

11. Loewi O. Über humorale übertragbarkeit der Herznervenwirkung. *Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere*. 1921; **189**(1): 239-42.

12. Dale HH, Dudley HW. The presence of histamine and acetylcholine in the spleen of the ox and the horse. *J Physiol*. 1929; **68**(2): 97-123.

13. Brock LG, Coombs JS, Eccles JC. The recording of potentials from motoneurones with an intracellular electrode. *J Physiol*. 1952; **117**(4): 431-60.

14. Eccles JC. *The Neurophysiological Basis of Mind. The Principles of Neurophysiology* New York, NY: Oxford University press, 1953.

15. Matsuo H, Baba Y, Nair RMG, Arimura A, Schally AV. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem Biophys Res Commun*. 1971; **43**(6): 1334-9.

16. Amoss M, Burgus R, Blackwell R, Vale W, Fellows R, Guillemin R. Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochem Biophys Res Commun*. 1971; **44**(1): 205-10.

17. Porter JC, Jones JC. Effect of plasma from hypophyseal-portal vessel blood on adrenal ascorbic acid. *Endocrinology*. 1956; **58**(1): 62-7.

18. Parlow AF. Bioassy of pituitary luteinizing hormone by depletion of ovarian ascorbic acid. In: Albert A, Thomas CC, eds. *Human Pituitary Gonadotropins* 1961: 300.

19. Fink G, Nallar R, Worthington WC, Jr. Demonstration of luteinizing hormone releasing factor in hypophysial portal blood of pro-oestrous & hypophysectomized rats. *J Physiol*. 1967; **191**(2): 407-16.

20. Fink G, Harris GW. The luteinizing hormone releasing activity of extracts of blood from the hypophysial portal vessels of rats. *J Physiol*. 1970; **208**(1): 221-41.

21. Sarkar DK, Chiappa SA, Fink G, Sherwood NM. Gonadotropin-releasing hormone surge in pro-oestrous rats. *Nature*. 1976; **264**(5585): 461-3.

22. Tsou RC, Dailey RA, McLanahan CS, Parent AD, Tindall GT, Neill JD. Luteinizing Hormone Releasing Hormone (LHRH) Levels in Pituitary Stalk Plasma During the Preovulatory Gonadotropin Surge of Rabbits1. *Endocrinology*. 1977; **101**(2): 534-9.

23. Carmel PW, Araki S, Ferin M. Pituitary Stalk Portal Blood Collection in Rhesus Monkeys: Evidence for Pulsatile Release of Gonadotropin-Releasing Hormone (GnRH). *Endocrinology*. 1976; **99**(1): 243-8.

24. Dierschke DJ, Bhattacharya AN, Atkinson LE, Knobil E. Circhoral oscillations of plasma LH levels in the ovariectomized rhesus monkey. *Endocrinology*. 1970; **87**(5): 850-3.

25. Caraty A, Orgeur P, Thiery JC. [Demonstration of the pulsatile secretion of LH-RH into hypophysial portal blood of ewes using an original technic for multiple samples]. *C R Acad Sci III*. 1982; **295**(2): 103-6.

26. Clarke IJ, Cummins JT. The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. *Endocrinology*. 1982; **111**(5): 1737-9.

27. Karsch FJ, Cummins JT, Thomas GB, Clarke IJ. Steroid feedback inhibition of pulsatile secretion of gonadotropin-releasing hormone in the ewe. *Biol Reprod*. 1987; **36**(5): 1207-18.

28. Caraty A, Locatelli A, Moenter SM, Karsch FJ. 9 - Sampling of Hypophyseal Portal Blood of Conscious Sheep for Direct Monitoring of Hypothalamic Neurosecretory Substances. In: Levine JE, ed. *Methods in Neurosciences*: Academic Press 1994: 162-83.

29. Ebling FJ, Lincoln GA. Endogenous opioids and the control of seasonal LH secretion in Soay rams. *J Endocrinol*. 1985; **107**(3): 341-53.

30. Caraty A, Locatelli A, Schanbacher B. [Augmentation, by naloxone, of the frequency and amplitude of LH-RH pulses in hypothalamo-hypophyseal portal blood in the castrated ram]. *C R Acad Sci III*. 1987; **305**(9): 369-74.

31. Caraty A, Locatelli A. Effect of time after castration on secretion of LHRH and LH in the ram. *J Reprod Fertil*. 1988; **82**(1): 263-9.

32. Knobil E, Plant TM, Wildt L, Belchetz PE, Marshall G. Control of the rhesus monkey menstrual cycle: permissive role of hypothalamic gonadotropin-releasing hormone. *Science*. 1980; **207**(4437): 1371-3.

33. Kaynard AH, Malpaux B, Robinson JE, Wayne NL, Karsch FJ. Importance of pituitary and neural actions of estradiol in induction of the luteinizing hormone surge in the ewe. *Neuroendocrinology*. 1988; **48**(3): 296-303.

34. Clarke IJ, Cummins JT, Jenkin M, Phillips DJ. The oestrogen-induced surge of LH requires a 'signal' pattern of gonadotrophin-releasing hormone input to the pituitary gland in the ewe. *Journal of Endocrinology*. 1989; **122**(1): 127-34.

35. Clarke IJ, Thomas GB, Yao B, Cummins JT. GnRH secretion throughout the ovine estrous cycle. *Neuroendocrinology*. 1987; **46**(1): 82-8.

36. Caraty A, Locatelli A, Martin GB. Biphasic response in the secretion of gonadotrophin-releasing hormone in ovariectomized ewes injected with oestradiol. *J ENdocrinol*. 1989; **123**(3): 375-82.

37. Clarke IJ, Cummins JT. Increased gonadotropin-releasing hormone pulse frequency associated with estrogen-induced luteinizing hormone surges in ovariectomized ewes. *Endocrinology*. 1985; **116**(6): 2376-83.

38. Goodman RL, Legan SJ, Ryan KD, Foster DL, Karsch FJ. Importance of variations in behavioural and feedback actions of oestradiol to the control of seasonal breeding in the ewe. *Journal of Endocrinology*. 1981; **89**(2): 229-40.

39. Moenter SM, Caraty A, Karsch FJ. The estradiol-induced surge of gonadotropin-releasing hormone in the ewe. *Endocrinology*. 1990; **127**(3): 1375-84.

40. Clarke IJ. Gonadotrophin-releasing hormone secretion (GnRH) in anoestrous ewes and the induction of GnRH surges by oestrogen. *Journal of Endocrinology*. 1988; **117**(3): 355-60.

41. Moenter SM, Caraty A, Locatelli A, Karsch FJ. Pattern of gonadotropin-releasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge. *Endocrinology*. 1991; **129**(3): 1175-82.

42. Hileman SM, Lubbers LS, Petersen SL, Kuehl DE, Scott CJ, Jackson GL. Influence of testosterone on LHRH release, LHRH mRNA and proopiomelanocortin mRNA in male sheep. *Journal of Neuroendocrinology*. 1996; **8**(2): 113-21.

43. Moenter SM, Brand RM, Midgley AR, Karsch FJ. Dynamics of gonadotropin-releasing hormone release during a pulse. *Endocrinology*. 1992; **130**(1): 503-10.

44. Moenter SM, Brand RC, Karsch FJ. Dynamics of gonadotropin-releasing hormone (GnRH) secretion during the GnRH surge: insights into the mechanism of GnRH surge induction. *Endocrinology*. 1992; **130**(5): 2978-84.

45. Evans NP, Dahl GE, Glover BH, Karsch FJ. Central regulation of pulsatile gonadotropin-releasing hormone (GnRH) secretion by estradiol during the period leading up to the preovulatory GnRH surge in the ewe. *Endocrinology*. 1994; **134**(4): 1806-11.

46. Halasz B, Gorski RA. Gonadotrophic hormone secretion in female rats after partial or total interruption of neural afferents to the medial basal hypothalamus. *Endocrinology*. 1967; **80**(4): 608-22.

47. Blake CA, Sawyer CH. Effects of hypothalamic deafferentation on the pulsatile rhythm in plasma concentrations of luteinizing hormone in ovariectomized rats. *Endocrinology*. 1974; **94**730-6.

48. Soper BD, Weick RF. Hypothalamic and extrahypothalamic mediation of pulsatile discharges of luteinizing hormone in the ovariectomized rat. *Endocrinology*. 1980; **106**(1): 348-55.

49. Boukhliq R, Goodman RL, Berriman SJ, Adrian B, Lehman MN. A subset of gonadotropin-releasing hormone neurons in the ovine medial basal hypothalamus is activated during increased pulsatile luteinizing hormone secretion. *Endocrinology*. 1999; **140**(12): 5929-36.

50. Moenter SM, Karsch FJ, Lehman MN. Fos expression during the estradiol-induced gonadotropin-releasing hormone (GnRH) surge of the ewe: induction in GnRH and other neurons. *Endocrinology*. 1993; **133**(2): 896-903.

51. Lee WS, Smith MS, Hoffman GE. Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrous surge of luteinizing hormone. *Proceedings of the National Academy of Sciences of the United States of America*. 1990; **87**(13): 5163-7.

52. Hoffman GE, Lee WS, Attardi B, Yann V, Fitzsimmons MD. Luteinizing hormone-releasing hormone neurons express c-fos antigen after steroid activation. *Endocrinology*. 1990; **126**(3): 1736-41.

53. Wang L, Guo W, Shen X, Yeo S, Long H, Wang Z, Lyu Q, Herbison AE, Kuang Y. Different dendritic domains of the GnRH neuron underlie the pulse and surge modes of GnRH secretion in female mice. *eLife*. 2020; **9**.

54. Evans NP, Dahl GE, Mauger D, Karsch FJ. Estradiol induces both qualitative and quantitative changes in the pattern of gonadotropin-releasing hormone secretion during the presurge period in the ewe. *Endocrinology*. 1995; **136**(4): 1603-9.

55. Evans NP, Dahl GE, Mauger DT, Padmanabhan V, Thrun LA, Karsch FJ. Does estradiol induce the preovulatory gonadotropin-releasing hormone (GnRH) surge in the ewe by inducing a progressive change in the mode of operation of the GnRH neurosecretory system. *Endocrinology*. 1995; **136**(12): 5511-9.

56. Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone H-J, Todman MG, Korach KS, Greiner E, Perez CA, Schutz G, Herbison AE. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron*. 2006; **52**271-80.

57. Christian CA, Glidewell-Kenney C, Jameson JL, Moenter SM. Classical estrogen receptor alpha signaling mediates negative and positive feedback on gonadotropin-releasing hormone neuron firing. *Endocrinology*. 2008; **149**5328-34.

58. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev*. 1999; **20**358-417.

59. Evans NP, Dahl GE, Padmanabhan V, Thrun LA, Karsch FJ. Estradiol requirements for induction and maintenance of the gonadotropin-releasing hormone surge: implications for neuroendocrine processing of the estradiol signal. *Endocrinology*. 1997; **138**(12): 5408-14.

60. Everett JW, Sawyer CH. A 24-hour periodicity in the "LH-release apparatus" of female rats, disclosed by barbiturate sedation. *Endocrinology*. 1950; **47**(3): 198-218.

61. Everett J, Sawyer C, Markee JE. A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cyclic rat. *Endocrinology*. 1949; **44 3**234-50.

62. Silveira MA, Burger LL, DeFazio RA, Wagenmaker ER, Moenter SM. GnRH neuron activity and pituitary response in estradiol-induced vs proestrous luteinizing hormone surges in female mice. *Endocrinology*. 2017; **158**(2): 356-66.

63. Christian CA, Mobley JL, Moenter SM. Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. *Proc Natl Acad Sci USA*. 2005; ***102***15682-7.

64. DePaolo LV, King RA, Carrillo AJ. In vivo and in vitro examination of an autoregulatory mechanism for luteinizing hormone-releasing hormone. *Endocrinology*. 1987; **120**(1): 272-9.

65. Caraty A, Locatelli A, Delaleu B, Spitz IM, Schatz B, Bouchard P. Gonadotropin-releasing hormone (GnRH) agonists and GnRH antagonists do not alter endogenous GnRH secretion in short-term castrated rams. *Endocrinology*. 1990; **127**(5): 2523-9.

66. Skinner DC, Caraty A, Evans NP. Does gonadotropin-releasing hormone in the cerebrospinal fluid modulate luteinizing hormone release? *Neuroendocrinology*. 1998; **67**(1): 37-44.

67. Padmanabhan V, Evans NP, Dahl GE, McFadden KL, Mauger DT, Karsch FJ. Evidence for short or ultrashort loop negative feedback of gonadotropin-releasing hormone secretion. *Neuroendocrinology*. 1995; **62**(3): 248-58.

68. Kasa-Vubu JZ, Dahl GE, Evans NP, Thrun LA, Moenter SM, Padmanabhan V, Karsch FJ. Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone. *Endocrinology*. 1992; **131**(1): 208-12.

69. Skinner DC, Evans NP, Delaleu B, Goodman RL, Bouchard P, Caraty A. The negative feedback actions of progesterone on gonadotropin-releasing hormone secretion are transduced by the classical progesterone receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; **95**(18): 10978-83.

70. Herbosa CG, Dahl GE, Evans NP, Pelt J, Wood RI, Foster DL. Sexual differentiation of the surge mode of gonadotropin secretion: prenatal androgens abolish the gonadotropin-releasing hormone surge in the sheep. *Journal of Neuroendocrinology*. 1996; **8**(8): 627-33.

71. Karsch FJ, Dahl GE, Evans NP, Manning JM, Mayfield KP, Moenter SM, Foster DL. Seasonal changes in gonadotropin-releasing hormone secretion in the ewe: alteration in response to the negative feedback action of estradiol. *Biol Reprod*. 1993; **49**(6): 1377-83.

72. Webster JR, Moenter SM, Barrell GK, Lehman MN, Karsch FJ. Role of the thyroid gland in seasonal reproduction. III. Thyroidectomy blocks seasonal suppression of gonadotropin-releasing hormone secretion in sheep. *Endocrinology*. 1991; **129**(3): 1635-43.

73. Dahl GE, Evans NP, Thrun LA, Karsch FJ. A central negative feedback action of thyroid hormones on thyrotropin-releasing hormone secretion. *Endocrinology*. 1994; **135**(6): 2392-7.

74. Goodman RL, Parfitt DB, Evans NP, Dahl GE, Karsch FJ. Endogenous opioid peptides control the amplitude and shape of gonadotropin-releasing hormone pulses in the ewe. *Endocrinology*. 1995; **136**(6): 2412-20.

75. Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ. Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge:

evidence that gonadotropes are not direct targets of kisspeptin in vivo. *Endocrinology*. 2008; **149**(4): 1951-9.

76. Caraty A, Lomet D, Sebert ME, Guillaume D, Beltramo M, Evans NP. Gonadotrophin-releasing hormone release into the hypophyseal portal blood of the ewe mirrors both pulsatile and continuous intravenous infusion of kisspeptin: an insight into kisspeptin's mechanism of action. *J Neuroendocrinol*. 2013; **25**(6): 537-46.

77. Padmanabhan V, McFadden K, Mauger DT, Karsch FJ, Midgley AR, Jr. Neuroendocrine control of follicle-stimulating hormone (FSH) secretion. I. Direct evidence for separate episodic and basal components of FSH secretion. *Endocrinology*. 1997; **138**(1): 424-32.

78. Steyn FJ, Wan Y, Clarkson J, Veldhuis JD, Herbison AE, Chen C. Development of a methodology for and assessment of pulsatile luteinizing hormone secretion in juvenile and adult male mice. *Endocrinology*. 2013; **154**(12): 4939-45.

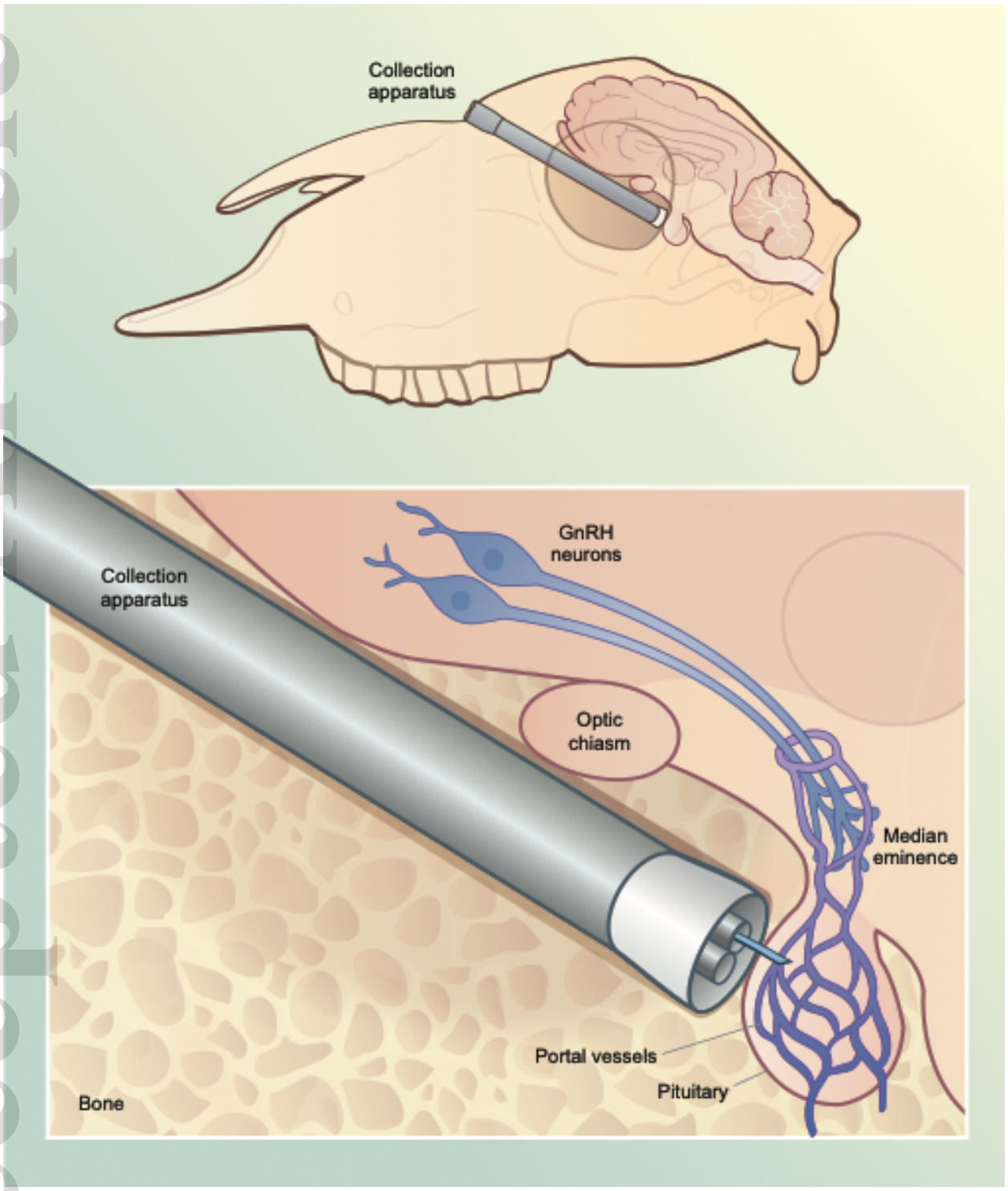

Figure 1. Illustration of the surgical approach taken and the final position of the collection apparatus ("gadget" or "gizmo") in a sheep's head to allow access to the hypothalamo-pituitary portal blood vessels for portal blood collection.