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Leap of Faith: Does serum luteinizing hormone always accurately reflect central reproductive neuroendocrine activity?

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Abstract

Function of the central aspects of the hypothalamo-pituitary-gonadal axis has been assessed in a number of ways including direct measurements of hypothalamic output and indirect measures using gonadotropin release from the pituitary as a bioassay for reproductive neuroendocrine activity. Here, methods for monitoring these various parameters are briefly reviewed and then examples presented of both concordance and discrepancy between central and peripheral measurements, with a focus on situations in which elevated GnRH neurosecretion is not reflected accurately by pituitary luteinizing hormone release. Implications for interpretation of gonadotropin data are discussed.

Keywords

GnRH; LH; kisspeptin; neurokinin B; pulse generator

Introduction

A central neuronal component to the control of anterior pituitary function for the regulation of reproduction was appreciated long before hormones from either location could reliably be measured or were even completely identified. Hypophysectomy stopped female reproductive cycles and decreased libido. Replacement via injection of pituitary extracts restored female cycles and libido [1]. A role for the hypothalamus was confirmed when electrical stimulation of this area was found to induce ovulation in female rabbits [2]. Rosalyn Sussman Yalow shared the Nobel Prize in Physiology and Medicine in 1977 for her work with Solomon Bernson to develop radioimmunoassays (RIA). Immunoassays opened a new world for endocrinology in which the dynamics of hormone release took a leading role because the sensitivity and relative simplicity of these methods made it possible to reliably analyze more frequent samples. In 1970, Dierschke et al., published serum concentrations of luteinizing hormone (LH) released from the anterior pituitary of primates as a function of sample frequency, demonstrating the now classic saw-tooth pattern of LH pulses [3]. That paper concluded that the rhythmic pulsatile release of LH might be due to “intermittent signals from the central nervous system”. This observation was made near the time the first

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hypothalamic releasing factors were being sequenced by brute force biochemistry. In 1971, Matsuo et al., published the sequence of what is now called gonadotropin-releasing hormone (GnRH, [4]), which is acknowledged to be the primary central factor regulating pituitary gonadotropin synthesis and release. Andrew V. Schally and Roger Guillemin, the heads of the laboratories that isolated the initial hypothalamic releasing factors for pituitary hormones, rounded out the Nobel Committee's selections for Physiology and Medicine in 1977.

Ernst Knobil and colleagues conducted classic ablation/replacement studies that demonstrated the effects of pulsatile vs. continuous GnRH on gonadotropin levels (Figure 1, [5]). In these studies, the medial basal hypothalamus was lesioned to eliminate endogenous GnRH release and then GnRH replaced intravenously in different patterns. The observations that administration of GnRH in a continuous manner, rather than mimicking the presumed pulsatile release pattern, decreased circulating LH and follicle-stimulating hormone (FSH) levels within a few days, and that this decrease was reversible upon return to pulsatile administration demonstrated the importance of the episodic nature of the central signal. This finding remains the basis for the current use of long-acting GnRH agonists to treat both precocious puberty and sex steroid-dependent cancers [6–8], vs. episodic administration for fertility restoration [9]. To this day, the generation of episodic hormone release in reproductive neuroendocrinology remains a topic of investigation and lively debate.

Direct monitoring of central neuroendocrine activity

The two methods most commonly used to directly monitor the activity of the central reproductive neuroendocrine system are multiunit activity (MUA) and sampling pituitary portal blood for GnRH (Figure 2). The first method, MUA is measured using an array of electrodes deployed in a brain area of interest. The electrodes record action potential firing from several neurons in their vicinity and peaks in this signal are generated when multiple neurons fire in a coordinated manner. MUA measurements originally in the monkey [10] and subsequently in other species [11–14] demonstrated that peaks in neuronal activity within the medial basal hypothalamus were typically highly correlated with LH pulses. The phrase "GnRH-pulse generator" was coined to describe this neuronal activity. Two drawbacks of MUA records are an inability to identify the cell type or types being recorded, and at least one degree of separation (release of GnRH itself) between the peaks in neuronal activity and release of LH.

The second method for directly monitoring reproductive neuroendocrine activity is measuring GnRH released into pituitary portal blood. Portal sampling bridges some of the gap between medialbasal hypothalamic MUA and LH release by directly monitoring GnRH release. This technically difficult approach of accessing pituitary portal vessels is required because GnRH has a very short half-life due to enzymatic destruction in the blood; further the small volume of portal blood is considerably diluted in the cavernous sinus and jugular blood. As such, GnRH cannot be measured in the peripheral circulation. This method was first done in the rat [15, 16] but the small size of this species mandated removal of the pituitary precluding simultaneous measurement of LH and limiting interpretable sampling to the periovulatory period when a large release of GnRH occurs. Sheep proved to be an

excellent model for this approach; the large size allowed multiple simultaneous samples from both pituitary portal and jugular blood in fully conscious animals, permitting direct correlation of the two measurements within the same animal [17]. High-frequency sampling of portal blood suggested GnRH release in sheep and MUA peaks in primates have a similar shape with a sharp onset to a peak, a high plateau below peak values, then a precipitous decline to baseline [18] (Figure 2). Portal sampling provides an excellent measure of the integrated output of GnRH at the median eminence; it is this GnRH release that regulates the pituitary gland. Of note, recent studies in *in vitro* preparations using methods that allow more localized monitoring of GnRH release have also identified secretion in the perisomatic region [19] and at the intersection of two GnRH neuron processes [20]. GnRH released in these areas likely serves neuromodulator functions that have been suggested in several studies [21–23], and may be under different regulatory control than neuroendocrine release of GnRH for pituitary regulation [24]. With regard to neuroendocrine release of GnRH, microdialysis and push-pull perfusion have also both provided more localized measurements of release near the median eminence [25–27]. The triple crown of simultaneous recording of MUA peaks or other electrical activity, GnRH release and LH release remains unclaimed; however the high correlation of the neuronal measurement, whether MUA or GnRH release, with LH pulses lead to a reasonable postulate that LH pulses can be used as a bioassay for central reproductive neuroendocrine activity.

Matches and mismatches between GnRH release and LH release

In many biological states, the assumption that LH pulses reflect GnRH release pattern is likely a valid one. It remains, however, an assumption. Discrepancies between these measures can affect the interpretation of results based on observation of LH release alone.

The preovulatory GnRH surge

One such mismatche comes during the preovulatory period, when the action of sustained elevated levels of circulating estradiol from the mature follicle(s) triggers one of the rare positive feedback events in physiology, the preovulatory GnRH surge [16, 29–32]. Direct measurements of the GnRH surge in sheep indicate it begins coincident with the LH surge, but that it persists for considerably longer duration (Figure 3A). Blockade of GnRH receptors before onset of the surge or at different points during the LH surge eliminates or shortens the LH surge, but not the GnRH surge, indicating GnRH is a prerequisite for the duration of the LH surge [33]. Although the existence of a surge mode of GnRH release in humans remains a topic of debate [34–36] an estradiol-induced GnRH surge has been observed in rhesus monkeys [29], suggesting that at least some old world primates exhibit this phenomenon; presence of at least an episodic pattern of GnRH is acknowledged to be necessary for generation of LH surges even in primates [37]. Of note with regard to mismatches between GnRH and LH, if a GnRH surge does not exist in humans, this would be an example of mismatch in which a sustained LH increase is a false positive for increased central reproductive neuroendocrine activity.

Potential central roles for GnRH beyond its established action to induce the LH surge may provide insight into the extended nature of the GnRH vs. the LH surge in sheep. Specifically, the duration of the GnRH surge is quite similar to that of proceptive sex

behavior in ewes. This suggests a possible role for extended duration GnRH release in mating. Indeed administration of GnRH antagonists after the LH surge is complete can reduce receptivity in ewes [21]. This additional biological action provides a physiological rationale for GnRH release that is unaccompanied by LH release. The time of ovulation appears more related to the onset of the LH surge [38], and ovulation does not require the full spontaneous LH surge [39], suggesting it is initiated by a relatively short duration of exposure to LH. In contrast, the behavior needed to fertilize an oocyte requires continued exposure to GnRH. These observations indicate that not all release from GnRH neurons is detected by monitoring pituitary hormone in the peripheral circulation, and that GnRH has additional roles beyond regulation of gonadotropes.

High-frequency episodic GnRH release

The clear mismatch in the duration of GnRH and LH release during the preovulatory surge is a once a cycle phenomenon in females, and is a quantitative rather than a qualitative change. Examples of mismatch when GnRH release is episodic and examination of LH alone produces a qualitatively different interpretation about central function may more substantially affect how we think about the hypothalamo-pituitary-gonadal axis. One example of this is illustrated in Figure 3 [32]. During the mid follicular phase (Figure 3B), five GnRH/LH pulse pairs are evident. In contrast, during the late follicular phase just before surge onset, this relationship is less clear and LH release alone does not provide an accurate portrait of GnRH release (Figure 3C). There are several possible explanations for this. First, the amplitude of GnRH release is lower and may less effectively produce clear increases in LH that can be detected. Second, the GnRH release frequency is slightly higher, providing less time for the clearance of LH. The longer half-life of LH would preclude complete clearance from the circulation, thus obscuring pulses. Third, estradiol modifies GnRH pulse shape [40] potentially altering pituitary responsiveness [41, 42]. Fourth, it is possible that the readily releasable pool of LH is depleted by high frequency input. Finally, a possible technical reason, specifically that removal of a portion of portal blood for GnRH measurement may diminish the signal at the pituitary so that well-coordinated LH pulses are not produced. There are other examples in which high frequency, clearly episodic GnRH release is associated with circulating LH levels that do not reflect this activity, including thyroidectomized ewes (Figure 4A, [43]), long-term castrate rams (Figure 4B, [44]), and following treatment with the broad-spectrum opiate antagonist naloxone in rams (Figure 4C, [45]). This latter example is particularly compelling because lower frequency GnRH pulses are accompanied by clear LH pulses (h −3 to 0 in Figure 4C) in the same animal in which high frequency GnRH is accompanied by apparently apulsatile LH release. Unlike the example in Figure 3B, C above, the shift in GnRH frequency in response to naloxone in Figure 4C is rapid, and the degradation of the LH pulse signal is not likely attributable to any of the above limitations of portal blood sampling. Taken together, these examples indicate LH is not always a reliable biomarker when GnRH release frequency is high.

The most recent example of a possible mismatch between GnRH and LH release comes from study of the development of GnRH release during the prenatal through adult period. This study used fast-scan cyclic voltammetry (FSCV) to monitor GnRH release in the median eminence in brain slices. This method takes advantage of the oxidation of GnRH on

a carbon fiber microelectrode that can be positioned within a relatively discrete brain region [20]. A GnRH-specific signal was detected with FSCV in the median eminence, and also in the preoptic area when electrodes were placed near the apposition of two identified GnRH fibers or that of a GnRH fiber and soma. No signal was detected in the median eminence of hypogonadal mice lacking GnRH, demonstrating specificity for this method for the GnRH decapeptide. Another recent study of cultured GnRH neurons also demonstrated release in the perisomatic region, using uptake of the fluorescent dye FM1-43 [19]. An advantage of these newer approaches is their ability to detect release within very localized regions; a disadvantage is that both are currently accomplished in an *in vitro* preparation of either cultured cells (FM1-43) or acutely prepared brain slices (FSCV). This precludes simultaneous monitoring of LH, introducing another potential caveat that must be considered. Of note in this regard, for adult male mice the frequency of median-eminence GnRH release detected with FSCV *in vitro* [47] is similar to that of LH pulse frequency *in vivo* [48, 49].

When FSCV was used to monitor GnRH release frequency locally in the median eminence in brain slices made from mice aged from embryonic day 18 (E18) through 1wk postnatal, somewhat unexpected results were obtained [47]. Very high frequency release was observed from E18 through 1wk of age (~5 release events/h on E18, ~15 events/h within 24h of birth, ~5 events/h at 1wk). Frequency then dropped to almost zero by 2wk of age. High-frequency release during the first week was further demonstrated to be vesicle-mediated and independent of the neuromodulator kisspeptin. These observations were initially surprising as LH was undetectable at these ages, similar to previous observations [50–52], despite pituitary expression of both the gonadotropin subunit genes and GnRH receptor [47]. Evaluation of LH release *in vivo* in response to a single injection of GnRH revealed no secretion at 1wk of age, when GnRH release frequency was high in the median eminence, but a marked increase in response to GnRH at 2wk of age, when GnRH release was essentially absent. Although the pairing together of the *in vitro* GnRH and *in vivo* LH measures in different mice of the same ages must be done with caution, one possible explanation for these observations is that high frequency endogenous GnRH release reduced the responsiveness of the pituitary gland with regard to LH release, which then recovered when GnRH release frequency dropped about 2wk postnatal. Of interest, pituitary follistatin mRNA levels were elevated in control mice at 1wk of age, declining thereafter [47]; follistatin mRNA is upregulated by high frequency GnRH [53, 54], adding further evidence that the high GnRH frequencies observed in brain slices at this age may also be received *in vivo* by the pituitary gland and affect its function even though this is not reflected in LH release. The LH levels in mice through these stages of development suggest a physiological situation in which GnRH frequency is sufficiently high to lead to pituitary shut down, similar to both continuous GnRH (Figure 1) and high frequency GnRH (3–5 pulses/h) in ablation/replacement studies [55].

In the above examples of pulsatile release, it is important to point out that the discrepancies are scenarios in which low LH does not accurately reflect high frequency GnRH, rather than low GnRH driving disproportionately high LH release. While the relatively long half-life of LH clearly contributes to obscuring of clear pulses in the circulation, there are other aspects

to consider. These include changes in pituitary responsiveness to GnRH as well as other neuroendocrine and peripheral factors (e.g., steroids) that co-vary under these experimental conditions and may play important roles in sculpting the LH output. This could be achieved in part through changes in the GnRH-receptor number or myriad post-receptor processes that are reviewed elsewhere [7, 56–59] Increases in LH sample frequency may help clarify pulses in instances in which this variable is limiting, but in the examples shown this is typically adequate. Alternatively use of assays for free alpha subunit, which has a much shorter half-life than full LH dimer, may be another way to determine if it is LH half-life alone that leads to a “blurring” of the LH pulse pattern [60]. FSH, which has a longer half-life than LH, has been ignored for this discussion, as it would be even more problematic in terms of reflecting central neuroendocrine activity.

If LH does not always reflect GnRH release, does the observation of low LH levels always indicate *lack* of GnRH release?

This is a question that is critical to sculpting our interpretation of many studies in which LH is the primary variable measured. Direct measurement of central neuroendocrine activity remains difficult. The increasing use of genetic manipulations to probe further up the hypothalamopituitary gonadal axis and study cells that are afferent to the GnRH neuron means that more steps occur between the manipulation and the release of LH. Further, many of these studies are done in small laboratory species because of the power of genetics, a trend that is likely to continue even as CRISPR/CAS9 methodologies make other species more accessible to genetic manipulation [61]. The small blood volume of many of these species means that investigators must attempt to interpret an episodic pattern of LH, and thus GnRH release, from a limited number of samples. Together the biological ‘distance’ of the manipulation from LH measurement and the relatively low resolution of this measurement can contribute potential inaccuracies in interpretation.

Studies of the kisspeptin neuronal population in the arcuate nucleus serve to illustrate different ways to interpret the same data set. Arcuate kisspeptin neurons have been postulated to be the source of GnRH pulse generation [62, 63]. These cells are also called KNDy neurons because of their coexpression of both neurokinin B (NKB) and dynorphin, two other neuromodulators thought to be important in fertility control. The anatomical connections of these cells with each other and with GnRH neurons, in combination with their expression of receptors for NKB and dynorphin but not kisspeptin receptors, which are expressed by GnRH neurons has lead to the following model. KNDy neurons act as an interconnected network that is activated by release of NKB and activation of NK3 receptors. This activation initiates kisspeptin release, which can induce GnRH release both at the soma and at the terminals [24, 64, 65], with the latter release leading to increased LH secretion. The activation of KNDy neurons is also thought to initiate subsequent dynorphin release to terminate their increased activity, kisspeptin release and eventually GnRH release. Repeated cycles through this series of events would lead to episodic activation of GnRH neurons, hence a “pulse generator”.

Many studies have tested various aspects of this model GnRH for pulse generation with a wide range of results depending on how studies were done. For example NKB or the

neurokinin 3 receptor agonist senktide have been reported to increase, decrease or have no effect on LH release [66–70]. While differences in species and endocrine milieux no doubt can explain some of the range of results, it is also possible that mismatch exists between central reproductive neuroendocrine actions and what the pituitary reports with LH release. Few studies have combined administration of KNDy peptides with direct measures of central reproductive neuroendocrine output. One study in goats serves to illustrate different directions of movement of a central response (MUA) and LH release. Wakabayashi et al., [71] monitored LH in response to NKB administered into the lateral ventricle of ovariectomized goats. NKB caused a decrease in LH levels and an apparent loss of the pulsatile pattern (Figure 6). Had this had been the only measurement made, a logical conclusion would have been that NKB reduced GnRH release. GnRH itself was not measured in this study but MUA peaks within the arcuate nucleus that were coincident with LH release were monitored. NKB caused a marked increase in the frequency of peaks in MUA spikes coincident with the drop in circulating LH. Thus at least one central reproductive measure was increased despite the drop in LH. One possibility is that NKB activated KNDy neurons and this caused a large release of dynorphin, shutting down KNDy neurons and subsequent GnRH release. Another is that NKB activated KNDy neurons causing a large release of kisspeptin that induced GnRH release, but at such a high frequency that pituitary output was blurred. Finally, it is possible that NKB itself directly activated GnRH release, which has been demonstrated to occur in male mice [72]. While these possibilities remain to be investigated, the simultaneous measurements of both the hypothalamic and pituitary levels of the axis opened the door to additional interpretations.

Another interesting example is from studies in which estrogen receptor alpha (ER α) was selectively deleted from kisspeptin-expressing neurons using targeted expression of *cre* recombinase, so-called KERKO mice [73]. Female KERKO mice exhibit early vaginal opening and elevated LH levels at 15 and 25 days of age, but no difference at 35 days of age (Figure 7). Despite similar single point LH values, KERKO animals did not exhibit estrous cyclicity. Kisspeptin neurons are postulated to mediate steroid feedback to the GnRH system, thus the early vaginal opening and elevated LH levels appeared consistent with a loss of negative feedback leading to a precocious activation of GnRH release. But is the subsequent drop in LH due to eventual failure of KNDy drive to GnRH neurons, or is it due to suppression of the pituitary in response to persistent high frequency GnRH release? This question cannot be answered from the present data.

Conclusion

While LH clearly does not always accurately reflect central reproductive neuroendocrine activity, particularly when the central system is driven at a high frequency subsequent to experimental manipulation, for most physiologic measures, it remains a good bioassay. Exciting new genetic approaches make it possible to activate the central system to extents greater than typical physiology. These approaches are of value in trying to understand the neuronal networks underlying control of fertility, but high levels of central activation can add complexities that need to be considered. As the field implements these exciting new methodologies, one challenge is to make use of output measures that reflect, as much as possible, the nuanced patterning of the reproductive neuroendocrine system. Increased use

of new low volume assays for LH to monitor pulse patterns in mice [49], and optogenetic methods for activating central neuroendocrine pathways in vivo [74] may help in this regard. Moving genetic studies into larger species as CRISPR/CAS9 becomes more standard is another possible approach. While we need to be cautious in drawing absolute conclusions about central function from examining only pituitary output, interpretation of results should continue to be pushed to creative limits, so long as speculation is identified as such. Acknowledging multiple alternative mechanisms and interpretations does not diminish the importance of scientific findings. Rather it strengthens its scholarly aspects and broadens thinking in the field.

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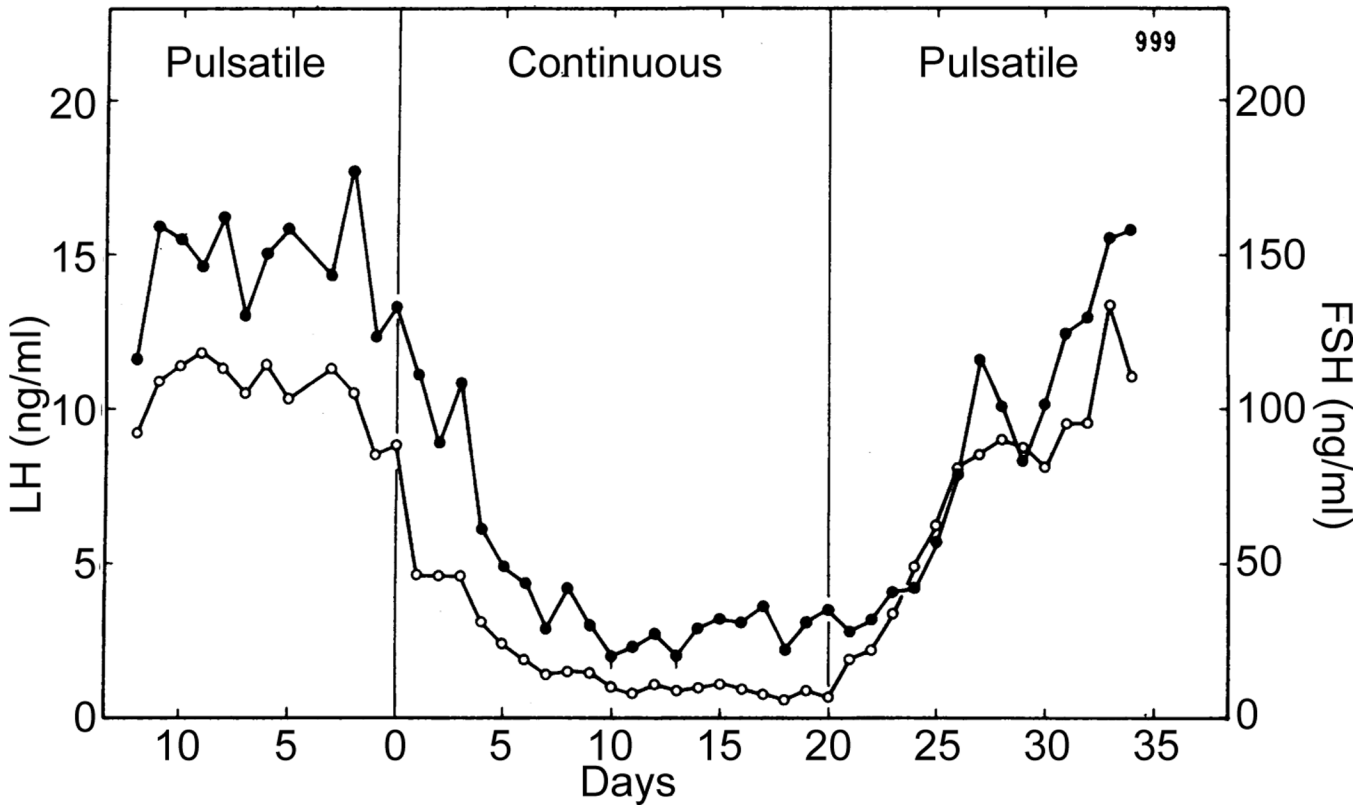

Figure 1.

Administration of hourly pulses of GnRH maintains typical levels of both LH (closed symbols) and FSH (open symbols) in ovariectomized rhesus monkeys. In contrast, continuous GnRH suppressed both hormones within about a week. Adapted from [5] with permission from AAAS

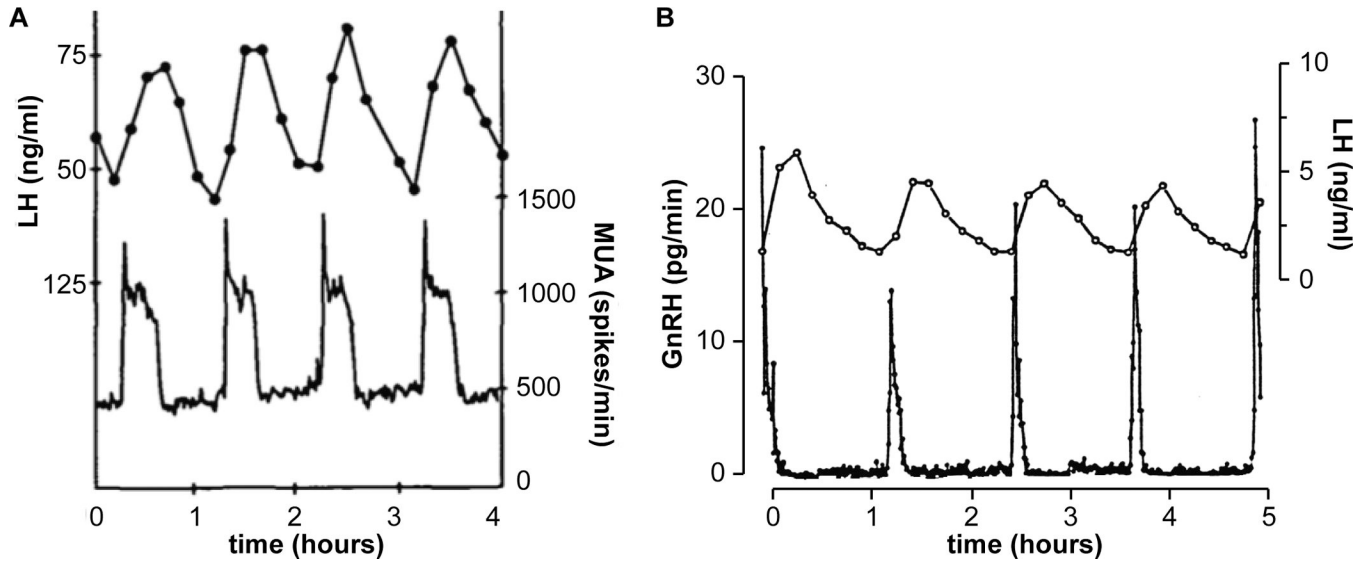

Figure 2.

Two methods for direct monitoring of reproductive neuroendocrine activity. Left, MUA measurements in the in the infundibular region of an ovariectomized rhesus monkey; peaks in MUA directly precede LH pulses. Adapted with permission from [28]. Right, simultaneous sampling of pituitary portal blood from an ovariectomized sheep at 30-sec intervals and jugular blood at 10-min intervals demonstrated a similar correlation between GnRH pulses and LH pulses. Adapted with permission from [18]. These figures have been scaled so that the X-axis is the same to facilitate comparison.

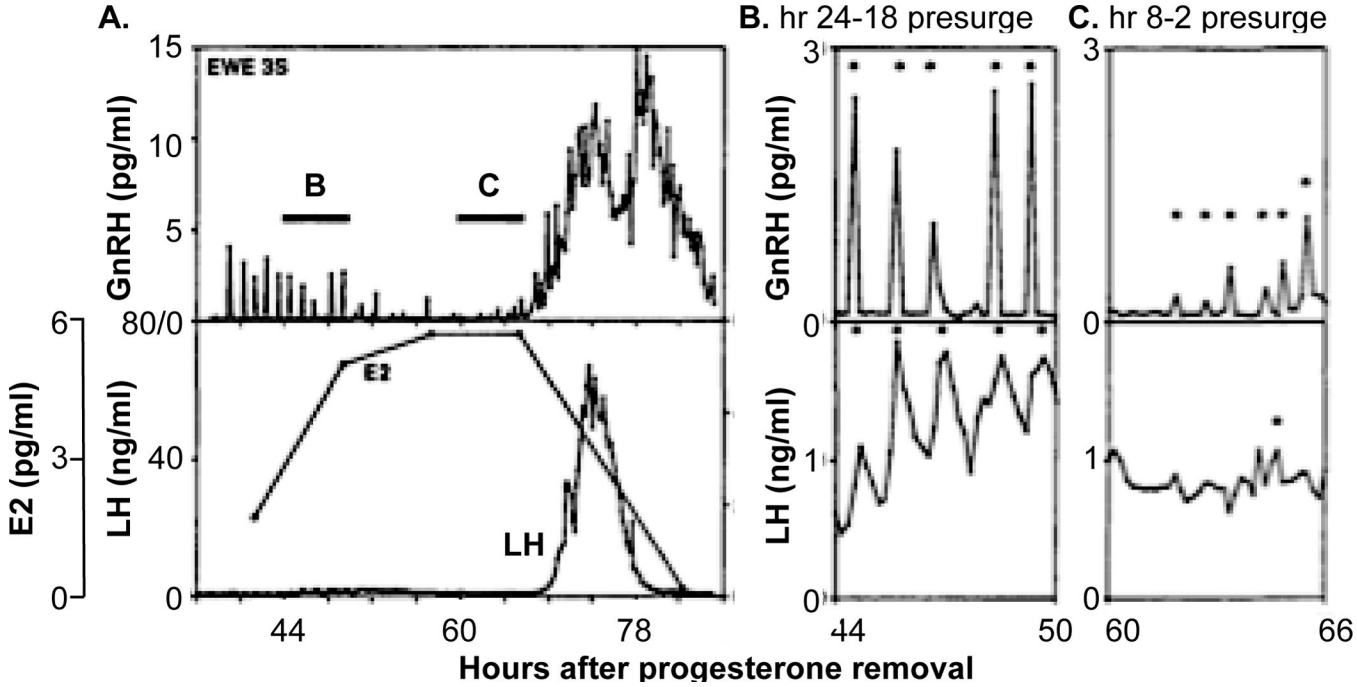

Figure 3.

A. GnRH (top), estradiol and LH (bottom) during an artificial follicular phase in sheep illustrating coincident onset of the GnRH and LH surges and prolonged duration of the former. B, C, pulsatile GnRH (top) and LH (bottom) release from the periods indicated by the bars labelled B, C in part A, * identified pulses. A-C adapted with permission from [32]..

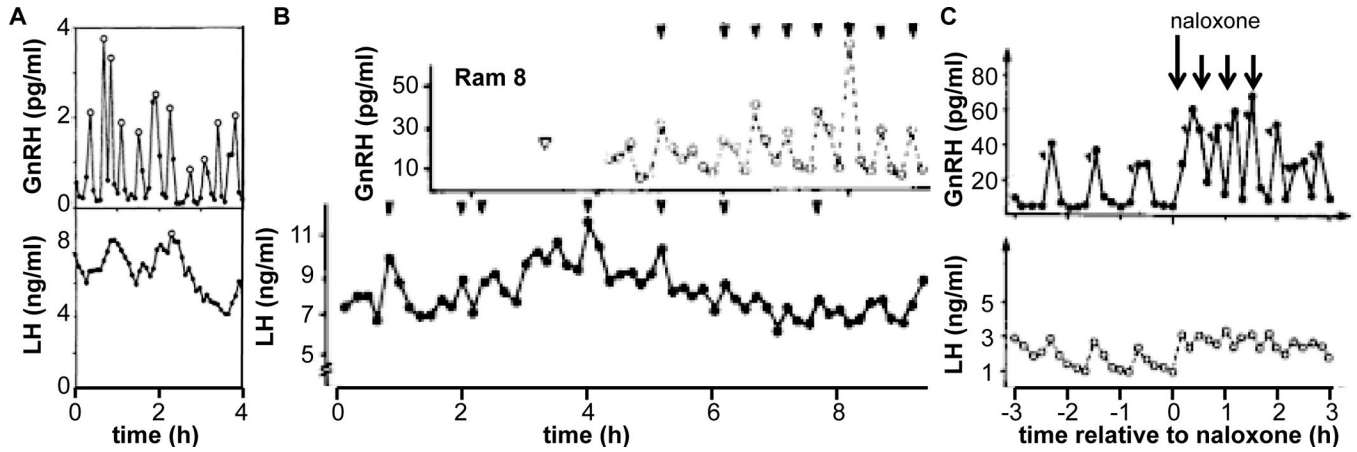

Figure 4.

LH is not always reliable biomarker of high frequency GnRH release. A. GnRH and LH release in a thyroidectomized ewe. Open circles mark pulses detected by the Cluster algorithm [46]. Adapted from [43]. B. GnRH and LH in a long-term castrate ram. Open arrowhead is time of portal vessel lesion for sampling; closed arrowheads mark detected pulses. Adapted from [44]. C. GnRH and LH release before, during and after treatment with naloxone (arrows, long arrow 100 mg, short arrows 50 mg dose). Angled arrowheads mark detected pulses. Adapted from [45].

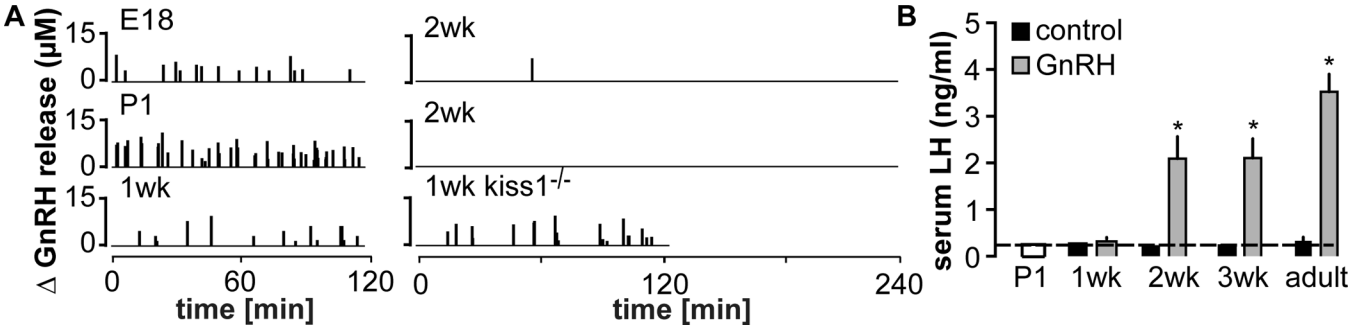

Figure 5.

High frequency GnRH release during late prenatal and early postnatal development does not generate pituitary LH release. A. Change in GnRH release in the median eminence at various ages. Vertical lines show GnRH release over time. B. Serum LH in control mice and 15 min after injection of GnRH. * P<0.05 relative to control of same age; P1 mice were not treated. Adapted from [47].

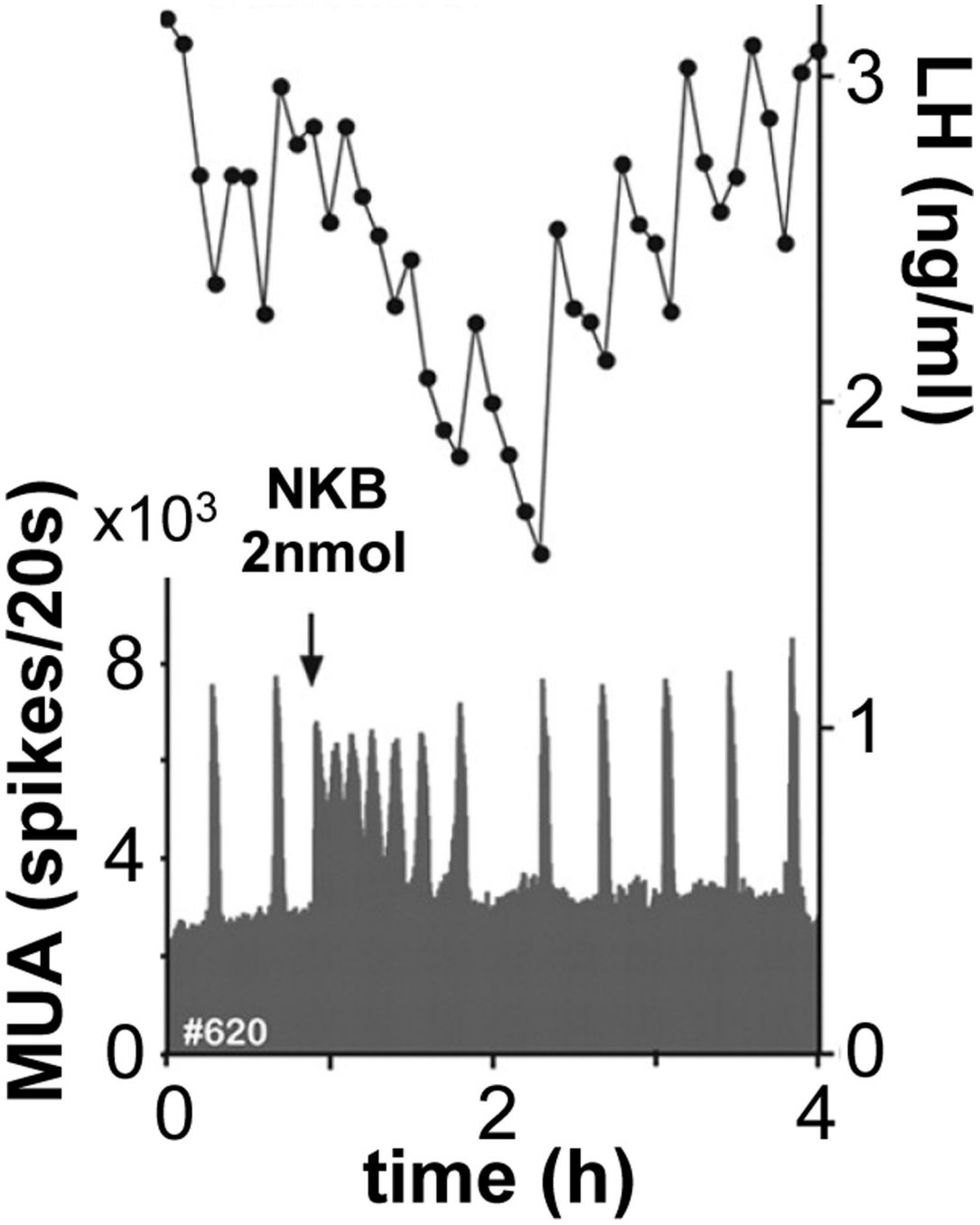

Figure 6.
Simultaneous measurements of MUA and LH reveal different directions of change. NKB increases the frequency of spikes of MUA but decreases serum LH levels. From [71] with permission.

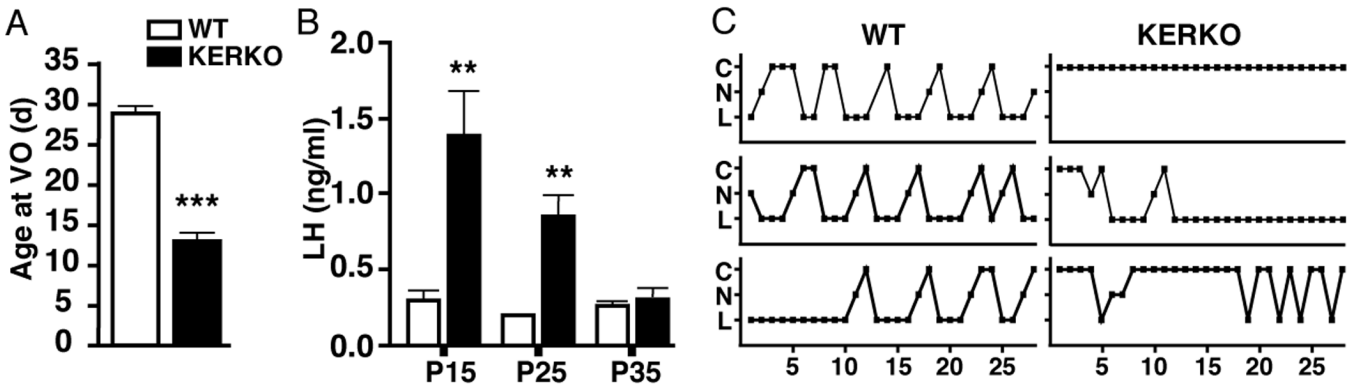

Figure 7.

Selective deletion of ERα from kisspeptin neurons causes early vaginal opening and elevated LH levels that subsequently return to control values but are not associated with estrous cyclicity. From [73] with permission.