Studies of the GnRH pulse generator. Developmental and location-specific regulation of GnRH secretion.

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A Dissertation presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Doctor of Philosophy

Neuroscience Graduate Program

University of Virginia
June, 2014

Abstract

The hypothalamo-pituitary-gonadal (HPG) axis governs reproductive function. Gonadotropin-releasing hormone (GnRH) neurons play a critical role in the central regulation of the HPG axis through the episodic release of the GnRH peptide to the hypophyseal blood. In addition to its neuroendocrine function, GnRH has been proposed to exert neuromodulatory role within the brain. GnRH neuron cell bodies are spread throughout the medial and basal hypothalamus and are found primarily in the preoptic area. They project their axons to the median eminence, where GnRH is secreted in a highly coordinated manner to regulate pituitary response. Interestingly, GnRH neuron physiological function appears to depend on their anatomical location. Therefore, GnRH secretion at different sites may be regulated by diverse mechanisms.

In this dissertation I provide new insights into the physiology of the GnRH neuronal system. These discoveries were enabled by a novel methodological approach for the direct detection of the GnRH release in the brain tissue. Previously the lack of such methods hindered the field from addressing important questions about mechanisms regulating GnRH secretion. Specifically, I adopt fast-scan cyclic voltammetry (FSCV) to measure both evoked and spontaneous GnRH release. I demonstrate via a series of control experiments that FSCV is a specific, sensitive tool for the monitoring of GnRH release with an excellent spatial and temporal resolution.

I applied the newly developed method to investigate mechanisms governing GnRH secretion in the preoptic area and the median eminence. These data provide evidence for significant regional differences in the role of action potential firing as well as modulation of GnRH release by kisspeptin and gonadotropin inhibitory hormone (GnIH). GnRH secretion in the median eminence requires firing activity in the neighboring

network that promotes kisspeptin release. Kisspeptin can act directly on the GnRH neuron terminals to increase intracellular Ca^{2+} concentration and trigger GnRH release. This is achieved via mechanisms involving mobilization of intracellular Ca^{2+} stores and Ca^{2+} influx through the cadmium-sensitive calcium channels. On the other hand, GnIH can prevent kisspeptin-induced GnRH release form the median eminence terminals. It acts most likely through the upstream network that is distal to the sites of kisspeptin action. In contrast to the median eminence GnRH release, in the preoptic area action potentials are not required for GnRH secretion and kisspeptin and GnIH targets tend to occur in the proximity to the GnRH release sites.

Next, the changes in the pattern of GnRH secretion from the median eminence and the development of pituitary response to GnRH during sexual maturation were investigated. Findings of these studies demonstrate that GnRH is released with an unexpectedly high frequency during perinatal life in male mice. Elevated frequency of GnRH release persists in one-week-old animals and appears to be due to the insufficient endogenous inhibitory signaling, but not an active excitatory drive. By two weeks of age GnRH release is almost completely abolished. This switch coincides with the pituitary gaining responsiveness to the GnRH signal. During the early life pituitary appears to be insensitive to the high activity of the GnRH system; a possible mechanism to prevent premature activation of the HPG axis.

Acknowledgements

To Sue, for being a great, supportive mentor that introduced some perturbations to my life and brought me to Ann Arbor.

To all the Moenter laboratory members I crossed my path with, for making time in this lab truly enjoyable.

To Tracy and Nadia, for excellent handling of all the bureaucratic and administrative matter, especially during and after the lab changed the institution.

To my committee members, for their invaluable input and guidance.

To Marcin, my fantastic partner in life, for faith, patience and support you give me.

To AJ, for bringing the *breath-bandha-drishti* triad, and a lot of fun, to my daily routine.

To Christine de Pisan, suffragettes, Elizabeth Garrett Anderson, Maria Skłodowska-Curie, Gregory Pinkus, Irena Krzywicka and Tadeusz Boy-Żeleński, Betty Friedan, Sheryl Sandberg and many, many others for laying foundations for truly free choices.

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Chapter 1

Introduction

GnRH neurons form the final common pathway for the central control of reproduction. As the key players in the process of GnRH pulse generation, they are an object of scientific interest for both neuroscience and reproductive biology. Broadening knowledge about their function in regulation of the activity of the hypothalamo-pituitary-gonadal (HPG) axis will allow better understanding of mechanisms underlying both normal fertility, as well as forms of hypothalamic infertility.

GnRH neurons release their decapeptide hormone into pituitary portal vasculature in an episodic manner (1–5). Pulse generation appears to be an integration point for various inputs and mechanisms involved in the CNS regulation of fertility, but the composition and mechanisms underlying synchronized production of GnRH pulses, the "pulse generator" remain poorly understood. The anatomy of the GnRH network imposes some crucial features of their function. GnRH neuron cell bodies are scattered throughout the medial ventral preoptic area and hypothalamus, with the biggest population residing in the preoptic area in most non-primate species (6). They send their axons to the lateral median eminence, where GnRH is secreted from densely packed terminals near the vasculature as a final output of the activity of the entire pulse generator. Interestingly, GnRH neurons fibers are also located within the preoptic area and hypothalamus and together with cell bodies are likely important targets for variety of inputs regulating GnRH neuron activity. This represents the classical function of dendrites as antennas gathering signals from other cells. However, the question of their active role in regulating the GnRH neuron function through either autocrine or paracrine mechanisms remains open and largely untested. In this regard, recent anatomical evidence shows complex interactions among GnRH neuron fibers (7) and current work in cultured GnRH neurons demonstrates GnRH release originating from both fibers and soma (8). In other neuroendocrine systems, dendritic release can be independent of terminal release and

plays a critical role in regulating cell function through local communication. Additionally, GnRH neurons can interact with their afferent network via mechanisms involving local neuronal/glial circuitry employing GABAergic, metabotropic glutamatergic and cannabinoid signaling (9,10). Finally, there is a preliminary indication that GnRH neuron fibers can propagate and potentially initiate action potentials, which in some GnRH neurons precede action potential firing detected in soma (11).

Some of the previously published work indicates the presence of GnRH receptor on prenatal GnRH (12) and adult neurons (13–15) as well as differential response of GnRH neuron electrical activity to GnRH peptide (13–15). It can be hypothesized in this regard, that GnRH neurons in the preoptic area can communicate with each other and their afferent network via mechanisms involving fiber and perisomatic release of GnRH and thus GnRH potentially acts as a neuromodulator in this region. In this dissertation, I will present a new methodological approach to study different functional aspects of the GnRH pulse generator, including its maturation throughout sexual development, and differential regulation of GnRH release depending on brain region. Furthermore, the role of known neuropeptidergic regulators of GnRH neurons activity, kisspeptin and gonadotropin inhibitory hormone (GnIH) in regulation of GnRH secretion in both the median eminence and preoptic area will be elucidated.

General background

GnRH is a decapeptide that was first purified and sequenced as an agent stimulating LH and FSH release by Schally et al. in 1971 (16). It had already been shown that LH is released in pulses (17), however the pattern of GnRH release responsible for production of this phenomenon remained unknown. A few years later, in 1978, Belchetz et al., demonstrated that intermittent, but not continuous GnRH infusion into rhesus monkeys

with hypothalamic lesions that ablated endogenous GnRH release could restore LH secretion (1). This experiment provided the first evidence for critical role of a pulsatile GnRH release pattern on proper downstream function of the pituitary; this was later confirmed by numerous studies done in different species and seems to be consistent for all vertebrates. The fundamental role of GnRH in fertility was shown, inter alia, by Cattanach et al. in 1977 (18). They described a mouse strain deficient in gonadotropin-releasing hormone, that is, a natural GnRH knockout. In this strain, called hpg mice, testes and ovaries fail to develop postnatally and mice are infertile. The evidence for a critical role of GnRH for human fertility was brought, among others, by Naftolin et al., in 1971 (19), when he showed the link between GnRH deficiency and a Kallmann's syndrome, a disease characterized by hypogonadism, which leads to infertility and anosmia (20). Further studies, together with development of GnRH-eGFP mice (6,21,22), provided deeper insight into understanding the role of GnRH neurons in regulation of fertility.

Anatomy and morphology of GnRH system

As mentioned previously, GnRH neurons in the central nervous system are a small population localized in the preoptic area and medial ventral hypothalamus (Figure 1-1). Because of their scattered location they are particularly difficult to study. During development, GnRH neurons migrate from the olfactory placode/vomeronasal organ of the olfactory system along vomeronasal nerve towards the forebrain, where they follow a branch of vomeronasal nerve to the hypothalamus and then send their processes to the median eminence (23,24).

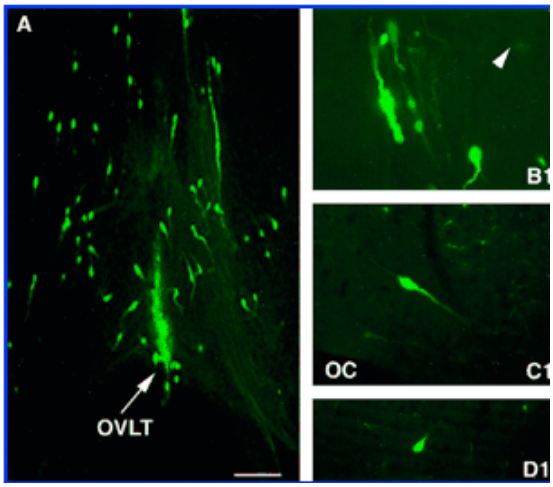

Figure 1-1. GFP-identified GnRH neurons. A. Section through the preoptic area, OVLT- organum vasculosum lamina terminalis. B1. Preoptic area. C1. Anterior hypothalamus over optic chiasm (OC). D1. Medial basal hypothalamus. From (6).

In mammals, the number of GnRH neurons varies from about 800 to 2500 (25). Morphologically GnRH neurons constitute a rather homogeneous population characterized mainly by flat spindle-shape soma with two processes on the opposite poles of cell body or by oval or triangle-shape cell body in case of multipolar cells (26).

Hypothalamo-pituitary-gonadal axis

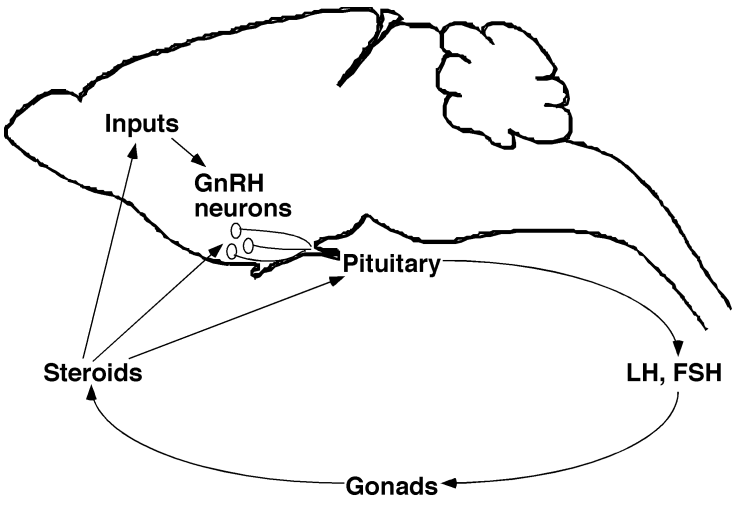

Figure 1-2. Hypothalamo-pituitary gonadal axis. Schematic overview of interactions between components of HPG axis including stimulatory role of GnRH on pituitary action and multiple feedback effects of gonadal steroids.

Fertility is regulated by feedback interactions among components of the hypothalamo-pituitary-gonadal axis (HPG). As mentioned previously, GnRH is released from GnRH neuron terminals in the ME, near the pituitary portal blood vessels and then stimulates release of gonadotropins, LH and FSH, from the anterior pituitary. In turn, LH and FSH regulate gonadal functions: gametogenesis and production of steroids. The latter feed back to the brain to provide both positive and negative regulation of GnRH neuron activity (27–30) (Figure 1-2). Steroid hormones act through steroid receptors, which are expressed in the brain (31–33), however only estrogen receptor type ß (ERß), which is not crucial for fertility but may play a modulatory role, appears to be expressed in GnRH neurons (34,35) to provide direct mechanism of feedback loop interactions within HGP axis. Estradiol as well as other steroids seem to regulate GnRH neuron activity through

indirect mechanisms involving GnRH neurons upstream network with both neuronal and glial components (27,36–41).

Ovulatory cycle

GnRH is secreted in a pulsatile manner and the pattern of these pulses is key to controlling pituitary activity. GnRH pulses vary in their frequency, amplitude and shape, but frequency appears to be the most important determinant of differential secretion of FSH and LH (1,2). It has been shown that high frequency pulses favor LH synthesis and release, whereas lower frequency pulses favor FSH synthesis and release (2,42). Changes in levels of LH and FSH are crucial for driving the ovulatory cycle (43). The human reproductive cycle can be divided into two phases, follicular and luteal, which are named for the main structures on the ovary and are separated by ovulation. In primates, the first day of menses marks the start of the follicular phase. During the follicular phase, the follicles containing the eggs develop and produce hormones. Ovulation occurs at the end of the follicular phase and the remaining cells of the follicle are converted into a corpus luteum, which produces hormones during the luteal phase (44–47); follicles present during the luteal phase in primates make little hormone due to their immature state but in non-primate species the continuous ongoing follicular development contributes to steroid milieu in the luteal phase (46–48). In mice we observe a similar scheme of a cycle, where ovulation (estrus) separates two distinct phases: one of final follicular maturation (diestrus and proestrus) and another of corpus luteum formation and regression (metestrus) (49). The major differences between a human and mouse ovulatory cycle, in addition to the time frame (human 28 days vs. mouse 4-5 days), are the number of follicles ovulated during a single cycle. In humans usually only one follicle matures, whereas in mice multiple follicles, even up to 20, could be recruited for ovulation (50). Additionally, the lifespan of the corpus luteum differs

significantly between these two species lasting for approximately 12-13 days in humans and only one day in mice (50). Schematic changes of plasma levels of hormones during the human cycle are shown in Figure 1-3.

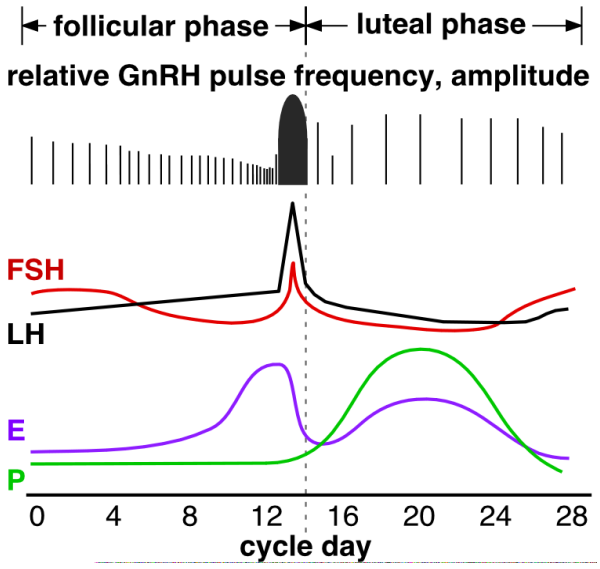

Figure 1-3. Human reproductive cycle. Schematic changes of hormone levels in accordance to changes in GnRH pulses pattern. Black vertical lines indicate GnRH pulses. FSH- follicle stimulating hormone, LH- luteinizing hormone, E- estradiol, P- progesterone. Dotted vertical line indicates timing of ovulation

During the early follicular phase GnRH pulse frequency is relatively low (48), which together with high amplitude favors FSH release. FSH stimulates follicular growth and selection of the dominant follicle. Due to a low level of progesterone, which is a result of the regression of the corpus luteum of the previous luteal phase, GnRH pulse frequency increases, which leads to an increase in the relative level of LH release (48). This switch in pulse pattern helps stimulate maturation of a dominant follicle and production of estradiol. The maturing follicles also produce inhibin, which specifically suppresses FSH release (51). Elevated estradiol inhibits the amplitude of GnRH pulses and can increase their frequency (52), which allows LH and estradiol to increase in parallel during follicular

phase, culminating in positive feedback of estradiol on GnRH pulse frequency. Moreover, estradiol induces elevated basal GnRH release between pulses (52). Sustained high levels of estradiol initiate positive feedback by inducing a surge of GnRH release and increasing the responsiveness of the pituitary to the increase in GnRH release (4). This combination of events results in surges in the release of both LH and FSH at the end of the follicular phase. The LH surge triggers an enzymatic cascade within the preovulatory follicle that leads to digestion of the outer follicle wall and ovulation (53), whereas the FSH surge is though to play a role in recruitment of the next follicles for development in subsequent ovarian cycles (54). Progesterone synthesized by corpus luteum inhibits GnRH pulse machinery by decreasing its frequency (55). If fertilization and implantation do not occur, regression of corpus luteum results in decreased progesterone levels (56), which liberates GnRH neurosecretory system from inhibition and initiates new preovulatory sequence of events.

GnRH pulse generator- comprehensive overview

The GnRH pulse generator is a critical mechanism that underlies cyclical changes in the HPG axis activity that helps drive the female reproductive cycle as well as male reproductive function, which is not cyclic, although it may undergo seasonal changes (57,58). Pulse generation appears to be an integration point for various inputs and mechanisms involved in the role of CNS in regulating fertility, but its composition and mechanisms underlying coordinated production of GnRH pulses remain poorly understood. In this section I will provide the overview of current knowledge regarding various patterns of episodic activity of the GnRH neuronal network in different models that were used to study this phenomenon. The models include immortalized GnRH neurons (GT-1 cells), primary neuronal cultures, as well as acutely dissociated adult cells and brain slices. All of the above have obvious limitations, which include the

effects of transformation and cells density in GT-1 cell cultures, possible inequality of primary cells to adult GnRH neurons, almost absolute disruption of physiological circuitry in the case of dissociated neurons and partial disruption in brain slices.

Episodic activity of GnRH neurons has been shown on different levels (e.g., pulsatile GnRH secretion, episodic bursts of action potentials) and in different time domains (low and high-frequency events). When discussing events occurring with frequencies of GnRH release it is important to mention that LH release is functionally coupled to pulsatile pattern of GnRH secretion. It has been shown in rodents, primates and sheep that each LH pulse detected in circulating blood is preceded by a GnRH pulse detected in pituitary portal vasculature. I will focus now on presenting GnRH neuron rhythms starting with events occurring at low frequencies comparable to those of GnRH release and then moving forward towards higher frequencies.

Experiments performed on cultured GT1 cells (immortalized GnRH neurons) provide evidence that rhythmicity in the time domain of hormone release (low-frequency) is intrinsic to networks of GnRH neurons. These cells demonstrate spontaneous pulsatile GnRH secretion in the absence of other cell types and pulsatile release is independent of macromolecular synthesis (59–62). GT1 cells also exhibit spontaneous increases in action potential firing activity with an interval between episodes of activity that is similar in range to GnRH secretion from native cultured GnRH neurons (63,64). These data are also supported by evidence for rhythmic synaptic vesicle release from networked GT1 cells (65,66). However, it has to be taken into account that the properties of GT1 cells can differ from native GnRH neurons in vivo because of their transformation and also higher density of GT1 cells in culture in comparison to GnRH neurons within a brain, where they are rather scattered and fewer in number. In addition to work in GT1 cells,

similar intervals between episodes of increased firing are also observed in individual GnRH neurons in brain slices (67).

Another level of rhythmicity of GnRH neurons is characterized by a high frequency mode, where activity periods are shorter than the typical period of secretory events. Bursts of action potentials repeating every few seconds have been observed in GT1 cells (68) as well as in GnRH neurons in brain slices (67,69) and after dissociation. GT1 cells, cultured embryonic GnRH neurons and GnRH neurons in slices also demonstrate tonic or irregular non-burst firing activity (6,21,69–71). The different (regular vs. irregular and burst vs. non-burst) firing patterns observed in GnRH neurons may be due to different experimental conditions, phase of the pulse cycle the GnRH neuron is in, or differences among in vivo conditions the cells were exposed to before recording. A small fraction (~1-2%) of GnRH neurons in mouse brain slices has also been shown to exhibit oscillations in membrane potential within both sub-threshold and supra-threshold potential values, where action potentials may be generated at the oscillation peak (72) with frequencies that in the context of the division introduced in this review would be considered high. When compared to other oscillating networks within CNS, however, these frequencies are rather low, and are more characteristic of endocrine pacemakers (73,74).

A much more prevalent type of high-frequency oscillations in GnRH neurons is observed in their intracellular calcium levels (shown on GT1 cells) (68); more recently dendritic calcium waves demonstrated (75). In addition to this high-frequency rhythmic activity both in GT1 cells and GnRH neurons in slices, with periods shorter than two minutes, cultured embryonic GnRH neurons have been shown to exhibit oscillations in intracellular calcium levels with periods around 8 minutes (76,77) that can be considered as intermediate-frequency events, and may represent the upper limit of GnRH pulse

frequency. In this regard, the highest GnRH pulse frequency observed in vivo is approximately one pulse every 12 min (78). Calcium waves with this intermediate period in the primate cells are synchronized with periods ~50 min, which is similar to the period of secretory activity in vivo in this species (Figure 1-4).

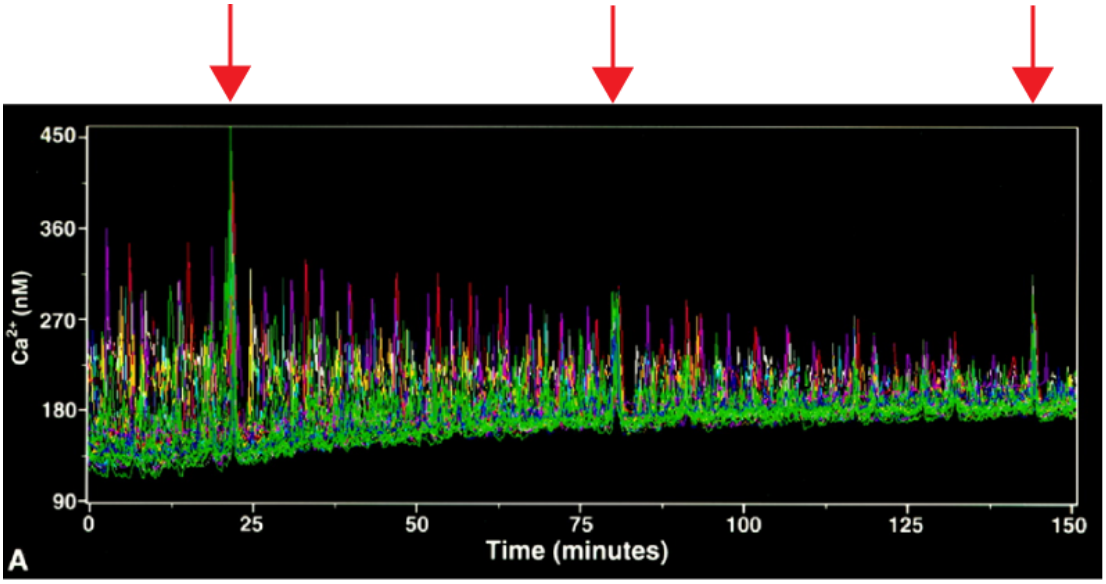

Figure 1-4. Synchronization of calcium waves in cultured rhesus monkey GnRH neurons. Each color is a trace from an individual cell. Red arrows indicate synchronization of calcium waves at 22, 81 and 143 minutes from time 0, demonstrating ~60 minutes intervals, which are similar to the pattern of GnRH pulses observed in that species adapted from (76).

This may suggest that calcium wave generation, and thus calcium signaling, is correlated with episodic secretion of GnRH, and calcium signaling could be involved in synchronization of the pulse generator (Figure 1-4). More recent work of Lee et al., shows that elevations in intracellular calcium in GnRH neurons are linked to burst firing; the latter underlies secretory activity in other neuroendocrine systems (79,80). Due to methodological difficulties such a link has not been tested in GnRH neurons yet. Once a method that allows for GnRH release detection with good temporal and spatial resolution

is available, the relationship between calcium waves and/or firing bursts of action potentials within single or multiple GnRH neurons and GnRH release could be established.

Mechanisms that underlie generation of activity in different frequency domains and at different levels of cellular output (from episodic firing to secretory events) are likely to be linked to two levels of network characteristics: intrinsic properties of GnRH neurons contributing to rhythmic activity as well as interactions with surrounding network that seems to provide regulatory inputs to GnRH neurons. These two features of GnRH pulse generator will be discussed in next sections.

GnRH pulse generator- concepts for coordination of GnRH neuron network

Vesicular release from neuron terminals is the result of a cascade of events often initiated by action potential firing. The resulting depolarization causes the influx of calcium through the channels in cell membrane, as well as release from intracellular calcium stores, allowing the molecular machinery involved in vesicle fusion to be activated. This type of mechanism is responsible for synchronous (coupled to action potentials) and asynchronous (immediately following action potentials) release, whereas spontaneous release, not coupled to action potential firing, doesn't require calcium signaling at all or uses calcium sensors extremely sensitive to even very small changes in its concentrations (81). Since GnRH is released, at least in the median eminence, in a very coordinated manner and in relatively large concentrations to the pituitary portal vasculature, it is likely AP-dependent release that should be considered as a major mechanism underlying GnRH secretion from their distal terminals. Coordination in terms of GnRH release in the median eminence must produce an intermittent pattern, however not all components of the GnRH network have to contribute to each episode of release,

or even to the entire episode of release. For example, to generate a GnRH pulse as long as few minutes (5), cells engaged in this process could release GnRH sequentially, with some of them releasing first, while others join later to facilitate the pulse throughout its duration. This idea can be supported by the experiments performed in GT1 cells, where asynchronous intermittent firing activity of multiple cells added up to a more regular pattern of increased episodic activity at the network level that occurred at intervals consistent with GnRH release (60–62,64,82).

Since GnRH neuron somata form a rather dispersed system within the hypothalamus, it is reasonable to consider the presence of other cell types contributing to the function of the pulse generator. GnRH neurons express different types of receptors for classical neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA) (83–85). It has been demonstrated by variety of studies that GABAergic transmission is among the major excitatory synaptic inputs to GnRH neurons (86–89). Additionally GnRH neurons are regulated by metabolic cues (90,91), steroid feedback (27,28) as well as time of day information (37).

Another potentially important component of network involved in regulation of GnRH neuron function are glia cells. Their traditional role in the CNS as a physical and nourishing supporters of neurons has been established as only one of many. Glia cells, especially astrocytes, but also radial glia cells, have been shown to communicate with various populations of neurons via so called gliotransmitters (e.g., prostaglandins, growth factors, cannabinoids). One function of astrocytes is modulation of neuronal excitability and synaptic transmission. This process first was shown to be mediated by glutamate (92–96), but more recently ATP and its derivative, adenosine, as well as endocannabinoids, have also been demonstrated to act as such modulators (97–100). Astrocytic coverage of synapses is another factor that can influence synaptic

transmission. It can control glutamate clearance and, in consequence, glutamate concentration and diffusion in extracellular space (101,102). Astrocytes are also involved in neuronal synchronization, of particular interest here in GnRH neurons, and this effect is mainly mediated by glutamate (103,104) and related to astrocytic calcium oscillations (105). Glia cells may also express receptors for steroids, and additionally synthesize pregnenolone and progesterone (106). This process may be induced in hypothalamic astrocytes by estradiol (107). This suggests that they can participate in hormone-mediated modulation of GnRH neuron activity. In this regard, glial apposition to GnRH neurons has been shown in ewes to correlate with seasonal changes in reproductive neuroendocrine function. During anestrous, glial ensheathment of GnRH neurons is significantly lower than during breeding season (108,109). Moreover, in rhesus monkeys, ovariectomy results in an increased ensheathment of glial processes to GnRH neurons in the medial basal hypothalamus and preoptic area. This effect is partially reversed by ovarian steroid replacement, which suggests that estradiol and/or progesterone may facilitate activation of GnRH neurons by decreasing their glial apposition (36,110,111). Recent studies on rats also demonstrate that aging might be another factor influencing the degree of glial escheatment on GnRH neurons in the median eminence (112,113).

Another important structural rearrangement in the median eminence involves neurovascular junctions and tanycytes. The terminals of GnRH neurons that reach median eminence are in a close apposition and are ensheathed by tanycytes (114–118) and the degree of that ensheathment changes throughout ovarian cycle (117,119) and may regulate the accessibility of GnRH neurons and thus released GnRH to capillary vessels. Additional evidence for glia-to-GnRH neuron communication comes from the

study showing effects of prostaglandins signaling on GnRH neurons electrical activity and membrane properties, which involve proper glial function (120).

The importance of GABAergic synaptic transmission to GnRH neurons applies not only to mediating steroid, metabolic and diurnal signals, but also is regulated by GnRH neuron feedback via local neuronal circuitries. Chu and Moenter (9) showed that action potential-like depolarization of GnRH neuron cell membrane leads to a brief (~10 s) reduction of frequency of GABAergic currents to that same neuron and is mediated by metabotropic glutamate receptor (mGluR) within a local circuit. Further, more recently published data suggest that this type of regulation of GnRH neuron activity is mediated by astrocytes and involves prostaglandin and cannabinoid receptor type 1 (CB1) signaling (121). Additionally, GABAergic transmission to GnRH neurons can be modulated by GnRH itself, providing additional evidence for other than terminal release of GnRH from GnRH neurons (14).

GnRH pulse generator function likely involves interactions between both the network of GnRH neurons, as well as between GnRH neurons and the surrounding network of other cell types. The GnRH neuronal circuitry as a system demonstrating a rhythmic activity is notably distinct from other such brain systems, which tend to be strongly synchronized, mainly due to direct connections through gap junctions and electrical coupling (for review see (122)). GnRH neurons do not seem to utilize these mechanisms. Even though some studies show the presence of connexins in cultured GnRH neuron and cytoplasmic bridges (123) between GnRH neurons in close apposition, only the limited dye transfer was observed (124), suggesting that electrical coupling is not the major mechanism regulating coordination between GnRH neurons. Additionally, no direct electrical communication between neighboring GnRH neurons has been observed in a functional electrophysiological studies (124), neither has fast synaptic transmission been observed

between pairs of GnRH neurons (72). It is important to point out that connexins are present in the median eminence, whereas dye transfer and electrophysiological experiments were performed in the preoptic area.

Other mechanisms of communication are possible since GnRH neurons have been shown to make both axosomatic, axodendritic and dendrodendritic connections with other GnRH neurons (7,109,125). There are a couple of candidates to be mediators between GnRH neurons, including GnRH itself, as well as glutamate. GnRH neurons express a functional GnRH1 receptor (13,14) and large-dense core vesicles containing GnRH are present in those cells (126). Interestingly, GnRH neuron response to GnRH is dose dependent (13,14). Small synaptic vesicles are also present in GnRH neurons (126) suggesting that they can also release conventional neurotransmitter such as glutamate or monoamines. Some GnRH neurons have been demonstrated to express vesicular glutamate transporter vGlut2 (127) and many of them have functional glutamate receptors (11,21,38,40). Since no direct fast synaptic or electric communication between GnRH neurons has been detected, at least when studied in existing experimental models, the question about alternatives for GnRH intra-network communication remains open.

In other neuroendocrine systems utilizing neuropeptides as major transmitters, including the widely studied and probably best-understood magnocellular system, two different types of neuropeptide release mechanisms with corresponding distinct functions have been described. Similar to GnRH neurons, magnocellular neurons project their axons for long distances (axons terminate in posterior pituitary, whereas cell bodies are found in supraoptic and paraventricular nuclei) and release vasopressin and oxytocin, unlike GnRH, directly in the pituitary. This process involves typical mechanisms for synaptic release of vesicles. Interestingly, the concentrations of those peptides in cerebrospinal

fluid (CSF) are typically higher than in blood and blood-brain barrier prevents them from re-entering the brain after release in the pituitary (128). This implies other source(s) of vasopressin and oxytocin in CSF than neurosecretion from pituitary axon terminals. In this regard, magnocellular neurons have been demonstrated to employ dendritic release, which is independent of terminal release, as a mechanism for autoregulation of their function and possibly for mediating the effects of these peptides in distal brain regions (129,130). The major differences between classical synaptic release and dendritic release of neuropeptides include the latter's activity-independent mechanism (does not depend upon action potential firing), it does not require high calcium concentrations and is often triggered by G-protein coupled receptor (GPCR) signaling (131,132). In addition to oxytocin and vasopressin, dendritic release of neuropeptides has been observed in locus coeruleus for galanin (133) and in the hippocampus for dynorphin (134). In this context GnRH neurons can be hypothesized to utilize similar mechanisms. Like peptides released by magnocellular neurons, GnRH can be detected in CSF in much higher concentrations than in hypophyseal blood and functional GnRH receptors have been found not only in GnRH neurons, but also on other cells in the hypothalamus and other brain regions (135,136). Further, more recent studies have brought some direct evidence for dendritic release from GnRH neurons. Research performed on GnRH neurons derived from monkey embryos demonstrates release of vesicles from both somatic sites and from processes, which may be dendritic (8). Additionally, our own experiments described in detail in Chapter 2 and 3, clearly indicate GnRH release in the preoptic area, which suggests possible somatic or dendritic release. Mechanisms that regulate GnRH release in different brain locations represent an important and interesting phenomenon to study. I will test the hypotheses that GnRH release in the preoptic area and median eminence can be differentially regulated, which may reflect different role for secreted GnRH between the two brain regions.

Development of the GnRH system

Pulsatile secretion of GnRH from GnRH neurons terminals in the median eminence is a well-described feature of adult brain, however its pattern before full sexual maturation has been poorly studied. In contrast, changes in anatomical features of GnRH system during development are broadly-investigated and demonstrate the complexity of the pulse generator. One of the unique characteristics GnRH neurons distinguishing them from other hypothalamic cells is that they originate outside of the brain in the vomeronasal organ, and more specifically, in the olfactory placode (137). They migrate then along the vomeronasal nerve into the brain following its route caudally to the hypothalamus (138), where most of GnRH neuron cell bodies are found in adult animals, and then send their terminals to the median eminence where GnRH exerts neuroendocrine function. This entire process requires an orchestration of variety of signals and cues to guide GnRH neurons to their proper localization. These include the whole range of adhesion molecules, guidance cues, neurotransmitters neuromodulators, growth and transcription factors (139–142). Alterations to proper function of those molecules can lead to developmental defects and thus reproductive disorders such as Kallmann's syndrome or other forms of hypogonadotropic hypogonadism (20,143–145).

Interestingly, GnRH receptor signaling has been proposed to be important in GnRH migratory process (146) and thus suggests that GnRH can be secreted in other regions than median eminence and in addition to classical neuroendocrine function could act as a neuromodulator during development and potentially later in life.

Once the anatomical organization of GnRH neurons is established, which in mice occurs by embryonic day 16.5 and includes GnRH neurons terminals reaching the median eminence (23,24), GnRH secretion to hypophyseal portal system can begin. The

evidence whether such release actually occurs is sparse and the general assumption in the field states that until puberty there is little to no secretory activity at the neuroendocrine level. Direct measurements of GnRH secretion from the median eminence at early stages in life in any species are currently unavailable; hence the postulate of inactive GnRH system is drawn from observed low levels of both gonadotropins and gonadal sex steroids measured before puberty (147–149). Although it has been demonstrated in adult sheep that GnRH pulses evoke subsequent LH secretion from the pituitary (4), and thus gonadotropins levels could be interpreted as an indicator of GnRH secretion when direct measurement of GnRH is not possible, no validation of this concept in neonatal or juvenile animals has been performed. Interestingly, some studies, both in vivo (2) and in vitro (150), indicate that high frequency GnRH pulses can desensitize pituitary response by essentially shutting down production and release of gonadotropins. This clearly suggests that under certain physiological conditions low LH levels may not indicate reduced GnRH secretion but rather the opposite. The lack of availability of relatively precise GnRH release detection tool that could be used in different species including rodents is an important obstacle in advancing research in this context.

Previous data reveal though, that the GnRH pulse generator before puberty can exhibit periods of increased activity. Both in humans and non-human primates so called mini-puberty has been described during neonatal life (151), which is characterized by increased levels of gonadotropins in both sexes. Elevated LH and FSH in humans persist for up to few months of life and then enter the period of relative tranquility to rise again once the pubertal process has been initiated (151,152). In mice a male-specific phenomenon has been observed during very early neonatal life (0-4 hr) when a brief elevation of testosterone occurs. It has been demonstrated in animal knockout models

though, that this is GnRH as well as kisspeptin-independent (153). Since these studies were performed in global non-inducible knockouts, it is still possible that compensatory mechanisms could be involved. Additionally, brief elevations of pituitary gonadotropins have been observed in some studies in rodents before the puberty (154,155) supporting the idea that GnRH pulse generator may not be completely inactive before the reproductive function is established. Details regarding the secretory activity of GnRH system before and during puberty remain elusive and further studies are necessary to uncover the nature of GnRH secretion during sexual maturation. We will address this question in Chapter 4.

Summary

Pulsatile release of GnRH that drives reproductive cycle is one of the most intriguing phenomena in neuroendocrinology. Due to the sparse nature of GnRH neuron distribution in the brain they are particularly difficult to study. The topology of the GnRH neuronal network does not suggest any obvious mechanisms that allow for their synchronized rhythmic activity. Certain methodological problems, which include lack of tools that enable for a real time monitoring of GnRH release from a single cell or a small group of cells, impede our capabilities to understand in details how GnRH pulse generator works. In this thesis dissertation I will introduce a new application of the electrochemical method Fast Scan Cyclic Voltammetry (FSCV) to measure GnRH content in mouse brain slices with time and spatial resolution sufficient to monitor levels of GnRH at sites of its release. I will then use that method to identify whether the median eminence is the only site of GnRH release within the brain. Further, FSCV will be used to study developmental changes in pattern of GnRH secretion from the median eminence of mice from perinatal period to adulthood. I will also examine the

mechanisms regulating GnRH release in different regions of the brain and discuss the implication of those studies for our understanding of GnRH pulse generator.

Chapter 2

Fast scan cyclic voltammetry as a novel method for detection of real-time gonadotropin-releasing hormone release in mouse brain slices

Katarzyna M. Glanowska, B. Jill Venton and Suzanne M. Moenter

Abstract

Pulsatile GnRH release is critical for the central regulation of fertility. There is no method allowing real-time GnRH detection in brain slices. We developed fast-scan cyclic voltammetry (FSCV) using carbon-fiber microelectrodes (CFME) to detect GnRH release and validate it using a biologically-relevant system. FSCV parameters (holding potential, switching potential and scan rate) were determined for stable GnRH detection *in vitro,* then optimized for GnRH detection in mouse brain slices. Placement of CFMEs in the median eminence (ME) near GnRH terminals allowed detection of both KCl-evoked and spontaneous GnRH release. GnRH release was also detected from GnRH fibers passing near GnRH soma and near fiber-fiber appositions in the preoptic area (POA). No GnRH signal was detected from CFMEs in the ME of *hpg* mice, which lack GnRH, or in regions not containing GnRH neurons in wild-type mice; application of exogenous GnRH produced a signal similar to that observed for spontaneous/evoked endogenous GnRH release. Using an established mouse model that produces diurnal variations in GnRH neuron activity, we demonstrated corresponding changes in spontaneous GnRH release in the median eminence. These results validate FSCV to detect GnRH in brain slices and provide new information on the sites and amounts of GnRH release, providing insight into its neuromodulatory functions.

Introduction

Pulsatile GnRH release is essential for fertility (1). GnRH is released from terminals in the ME near pituitary portal vessels. Modulation of GnRH pulse frequency is required for the differential release of luteinizing hormone (LH) and follicle-stimulating hormone from the pituitary that is crucial for driving female reproductive cycles (2). Abnormal GnRH-pulse patterns can cause infertility (156). In addition to release at the ME, GnRH may

act as a neuromodulator in local circuits (13–15,157,158), but release has not been demonstrated from other brain regions *in situ*.

Recent work has examined the pattern of action potential firing from GnRH neurons in brain slices and primary cultures (27,28,37,69,159–161). There is no empirical link, however, between the firing pattern of GnRH neurons and the pattern of release produced. This is attributable to a lack of a method that allows for GnRH release detection in preparations used for electrophysiology.

GnRH contains electrochemically active amino acids, in particular tryptophan and tyrosine. Electrochemically-active species can be detected and identified using fast scan cyclic voltammetry (FSCV) based on their oxidation and reduction properties. This method has been used to detect small molecule neurotransmitters, such as monoamines (162). Our objectives were to adapt FSCV to enable GnRH detection in brain slices, to determine where release occurs, and to test the ability of FSCV to detect biologically-relevant changes in GnRH release.

Materials and Methods

CFME fabrication CFMEs were fabricated as described (163). Carbon fiber (T-650, generous gift from Cytec Engineering Materials) was aspirated into a capillary glass tube (1.65/1.1mm OD/ID, World Precision Instruments), which was pulled (PE-21 (Narishige) or P-97 (Sutter Instruments)). The fiber was cut to 40-60μm and sealed to the glass using Epoxy Resin 858 (Miller-Stephenson) and 14% (w/w) m-phenylenediamine (Fluka). Electrodes were incubated overnight at room temperature, baked 2h (100°C) and then overnight (150°C). Before experiments, electrodes were washed ≤10min in isopropanol, and then filled with 1M KCl.

FSCV in vitro Chemicals were from Sigma Chemical Company unless noted. Data were collected using a custom-modified Dagan ChemClamp potentiostat and Tar Heel software (gift of Mark Wightman) or an extended range (±2V) EPC10 patch-clamp with PatchMaster (HEKA Elektronic) with Demon Voltammetry (Wake Forest University). *In vitro* experiments were done in pH 7.4 Tris buffer containing (in mM): 15-tris(hydroxymethyl)aminomethane, 140 NaCl, 3.25 KCl, 1.2 $CaCl_2$, 1.25 NaH_2PO_4, 1.2 $MgCl_2$, and 2.0 Na_2SO, as described (164). CFMEs were calibrated *in vitro* using a flow-injection apparatus. Three-second injections of compounds of interest (1μM tryptophan or 5μM GnRH in Tris buffer) were used to mimic fast concentration changes expected in biological systems. For both tryptophan and GnRH, the waveform parameters (holding and switching potentials, and scan rate) were optimized towards detection of the single, specific oxidation peak.

Animals Transgenic mice expressing green fluorescent protein (GFP) under the control of the GnRH promoter (GnRH-GFP) (6) were used for spontaneous and evoked release studies. To control for specificity, hypogonadal (*hpg*) GnRH-GFP-hpg mice (*hpg*-GnRH-GFP, generous gift of Drs. Ursula Kaiser and John Gill)(165); these mice lack GnRH peptide (166). Tac2-GFP transgenic mice (015495-UCD/ STOCK Tg (Tac2-EGFP)381Gsat, Mouse Mutant Regional Resource Center) were used as a positive control for detecting evoked GnRH release without GFP identification of terminals.

Mice were housed under a 14:10hr light/dark photoperiod with Harlan 2916 chow and water available *ad libitum*. Adult females (42-60d) were used. Ovariectomy (OVX) was performed under Isoflurane (Burns Veterinary Supply) anesthesia. Bupivicaine (0.25%, 7μl per surgical site, Abbott Labs) was applied to surgery sites to reduce postoperative pain and distress. During OVX, mice received subcutaneous Silastic (Dow-Corning) implants containing 0.625μg 17β-estradiol (E) in sesame oil with or without progesterone

(P) implants (2.5mg, Innovative Research of America). OVX+E mice, which generated daily LH surges (37), were used 2-4d post surgery; OVX+E+P mice were used 5-10d post surgery. All procedures were approved by the University of Virginia Animal Care and Use Committee and the University of Michigan University Committee on the Use and Care of Animals.

FSCV in brain slices Brain slices were prepared as described (9,67). All buffers were bubbled with 95%O2/5%CO2 ≥15min before usage. Sagittal (OVX+E+P and OVX+E AM vs. PM) or coronal (*hpg* and Tac2 mice) 300μm brain slices were cut using a Vibratome 3000 (Ted Pella) in ice-cold sucrose saline containing (in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 glucose, 1.25 NaH_2PO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Slices were incubated 30 min at 30-32°C in a 1:1 mixture of sucrose saline and artificial-cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, and 10 D-glucose, pH 7.4, then transferred to 100% ACSF and incubated 30-300min at room temperature before study.

For GnRH detection, potential was continuously scanned from 0.5-1.45V at 400V/s every 100ms (Figure 2-1A). CFMEs were stabilized for 15min before collecting data. Signals arising from spontaneous release were recorded for 20min either in the ME or POA, and then secretion was evoked by a 20mM KCl ACSF. For biological validation of hypothesized changes in GnRH release with time of day in OVX+E mice, GnRH release was monitored continuously for 1h with CFMEs placed either near GnRH neuron terminals in the ME or in the POA near fiber-soma appositions.

CFMEs were placed in the cortex or hippocampus, away from GnRH soma/processes, to perform controls. To document the GnRH-generated FSCV signal in the brain slice environment, 5μM GnRH was applied near CFMEs. To control for specificity, kisspeptin

(5μM), which may be released in the ME, was applied near CFMEs in the cortex/hippocampus. In addition, CFMEs were placed in the ME of hpg-GnRH-GFP mice and Tac2-GFP mice, and KCl-evoked signal examined. Following all recordings, CFMEs were calibrated in 5μM GnRH *in vitro*.

Data analysis Data were analyzed using Tar Heel or Demon software (Wake Forest University Health Sciences) as described (163). Cyclic voltammograms (CVs) were background-subtracted by averaging 10 background scans. To verify the identity of a spontaneous release peak as GnRH, five control CVs collected after GnRH was injected into a slice were averaged. Each putative GnRH CV was correlated with this average and was considered to be GnRH if $R^2 \geq 0.8$. This threshold was set to allow for some electrode variability. 96% of CVs passed this test; those with $R^2 < 0.8$ were always flanked by events with robust correlations. For the daily surge experiment (OVX+E animals), data were binned at 1-min intervals to facilitate evaluation of the pattern of release. Maximum change in GnRH concentration for each 1-min bin was plotted vs. time (Figure 2-2A). Event duration was the number of consecutive bins in which a change in GnRH was detected; events separated by a single 1-min bin without detection of GnRH were considered to be single events. Changes in GnRH concentration were estimated based on calibration in 5μM GnRH. Data are expressed as mean±SEM and analyzed using ANOVA with Bonferroni *post hoc* test (GraphPad Prism); $p<0.05$ was considered significant.

Results

Detecting tryptophan and GnRH in vitro. FSCV parameters were optimized for stable *in vitro* detection of tryptophan (1μM). Holding potential (potential at the start 0.4-0.6V), switching potential (potential at the peak 1.1-1.3V), scan rate (50-500V/s) and

waveforms were varied within the ranges indicated. Tryptophan is known to foul the electrode under some conditions (167), so FSCV parameters were chosen to minimize loss of sensitivity and produce consistent cyclic voltammograms (not shown). The oxidation peak for 1μM tryptophan was 0.9V whereas the oxidation peak was shifted to higher voltages (~1.25V) for 5μM GnRH (Figure 2-1A). This shift in peak may be attributable to slower kinetics of tryptophan oxidation when it is incorporated into the peptide. The peak signal obtained for 5μM GnRH with the optimized waveform of 0.5V to 1.45V at 400V/s (voltage protocol Figure 2-1A) was 2.5±0.1nA (n=5).

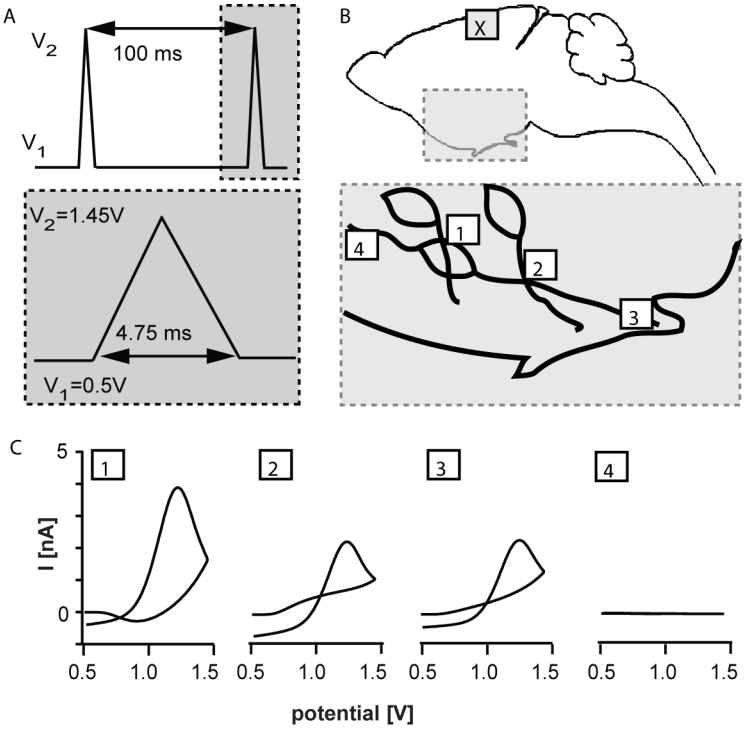

Figure 2-1. A. FSCV protocol used to detect GnRH. V1 is holding potential, V2 is switching potential. Grey area is expanded below. B. Illustration of recording sites. *Top* sagittal section; grey area is expanded below. X indicates location of CFMEs for exogenous GnRH application. *Bottom* sites of GnRH release 1-GnRH soma-fiber contact (POA), 2-GnRH fiber-fiber contact (POA), 3-GnRH terminals in the ME, 4-single GnRH fiber (POA). C. Example CVs taken at peak current of GnRH signal detected in locations indicated in B, R^2 is in upper right corner.

FSCV in mouse brain slices. We next recorded from GFP-identified GnRH neurons in brain slices (6). Female OVX+E+P mice were used as this treatment produces low endogenous GnRH neuron activity (28). Because FSCV detects changes in concentration rather than absolute concentration, a low baseline was desired for initial tests. CFMEs were positioned near GFP-identified terminals in the ME. After stabilization, data were collected for 20min to reveal any spontaneous release, followed by treatment with 20mM KCl to evoke release. Both spontaneous and evoked GnRH release were detected (Figure 2-2C, D, Table 2-1). The peak oxidation potential was shifted to slightly higher voltages in the brain slice (1.32±0.02V) than was observed in vitro (1.20±0.05V). Although the current flow indicates oxidation, the peak occurs during the return of the voltage from switching potential to holding potential. This is likely attributable to slower electron transfer kinetics in electrodes placed in tissue; this was expected as the surrounding milieu for the peptide is different. To test if changes in milieu between pure buffer and the brain slice would cause a similar shift in cyclic voltammogram, CFMEs were positioned in the cortex or hippocampus and 5μM GnRH locally applied. The signal obtained (Figure 2-2B, Table 2-1) was similar (R^2>0.8) to that for spontaneous and evoked release of GnRH detected by CFMEs in the ME (Figure 2-2C, D, Table 2-1). The signal observed for mammalian GnRH differs from that of teleosts (168), likely due to different materials of the CFME and amino acid sequence.

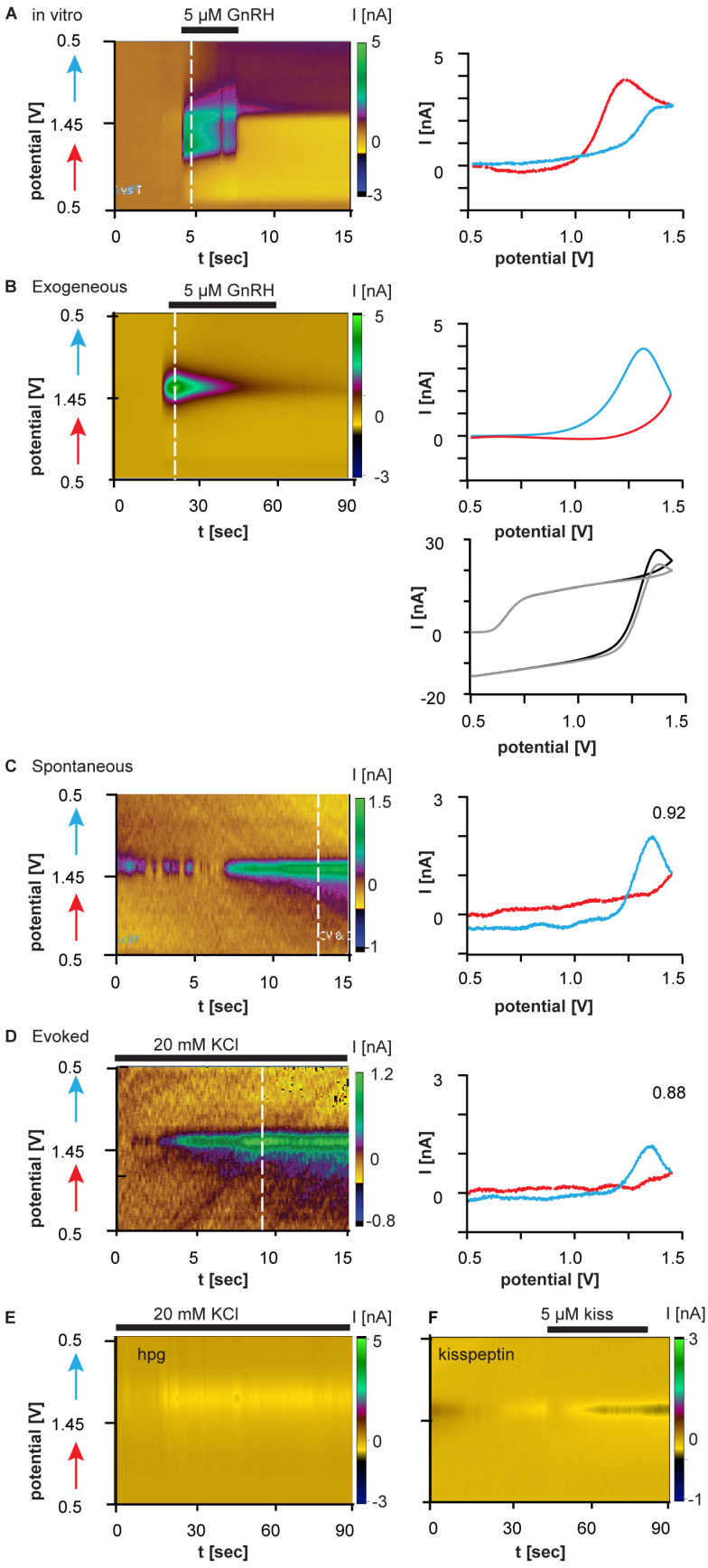
A in vitro
5 μM GnRH
I [nA]
potential [V]
t [sec]
B Exogeneous
5 μM GnRH
I [nA]
potential [V]
t [sec]
C Spontaneous
I [nA]
0.92
potential [V]
t [sec]
D Evoked
20 mM KCl
I [nA]
0.88
potential [V]
t [sec]
E
20 mM KCl
I [nA]
hpg
potential [V]
t [sec]
F
5 μM kiss
I [nA]
kisspeptin
t [sec]

Figure 2-2. FSCV recordings of evoked and spontaneous GnRH release monitored by CFMEs in brain slices. A,C,D were from CFMEs in the median eminence; B,F, from CFMEs in cortex. *Left* panel in A-D shows electrochemical current in a color heat plot as a function of voltage and time. Red arrows show the forward voltage scan and blue arrows show the reverse scan. Dotted white lines indicate time for which cyclic voltammogram plotted on the right. *Right* panel in A-D shows a background-subtracted cyclic voltammogram. A. *In vitro* GnRH signal. B. Exogenous GnRH applied in cortex generates a similar cyclic voltammogram to endogenous GnRH (C, D). *Lower right panel* in B shows raw background voltammograms in the presence (black trace) and in the absence (grey) of GnRH. C. Spontaneous GnRH release in GnRH-GFP mouse. D. KCl-evoked GnRH release in GnRH-GFP mouse. For C and D, R^2 is in upper right corner. E. No GnRH release evoked by KCl in the ME of *hpg* mice, which lack GnRH. F. Exogenous kisspeptin applied in the cortex does not generate a signal.

Table 2-1. Summary of control experiments.

location	genotype	treatment	n	nA
cortex/hippocampus	GnRH-GFP	20mM KCl	5	0
cortex/hippocampus	GnRH-GFP	5μM GnRH	10	1.6±0.4
cortex/hippocampus	GnRH-GFP	5μM kisspeptin	3	0
Median eminence	hpg	20mM KCl	9	0
Median eminence	Tac2-GFP	20mM KCl	7	3.3±0.7

The presence of tryptophan and tyrosine in many proteins raises an obvious question of specificity. In this regard, tryptophan and tyrosine are hydrophobic, tending to be on the inside of folded proteins (169,170), thus minimizing interference from large proteins as these amino acids are inaccessible for oxidation. When tryptophan and tyrosine are accessible as in smaller peptides like GnRH, surrounding amino acids will influence the oxidation profile, thus it should be unique for each substance. We tested specificity in several ways (Table 2-1, Figure 2-2E, F). First, no GnRH signal was observed in brain regions lacking GnRH peptide even in the presence of high potassium to evoke release (data not shown). Second, the small tryptophan-containing peptide kisspeptin-10 (1-5μM) did not produce a signal similar to that of GnRH (Figure 2-2F, R^2=0.01). Third, no GnRH signal was evoked by high potassium from the ME of *hpg* mice, which have no detectable GnRH peptide despite a normal distribution of GnRH and other neuroendocrine neurons (Figure 2-2E) (165). In contrast to the lack of GnRH signal in ME from *hpg* mice, typical potassium-induced GnRH release was recorded from the ME of control mice, even when CFME placement was guided solely by anatomy (i.e., no GnRH-GFP signal, Table 2-1, R^2<0.8). Finally, it should be noted that although voltammetry is used to detect small neurotransmitters such as catecholamines, these neurotransmitters oxidize at much lower voltages, near the holding potential in these experiments (171). Thus no signal would be expected from these substances as they are already oxidized. Together these data suggest the signal is specific for GnRH.

Sites of endogenous GnRH release. We next tested sites of GnRH release. CFMEs were positioned: (**1**) in the POA between GnRH fibers and somata (**2**) in the POA where GnRH fibers crossed one another and (**3**) in the ME among GnRH terminals and (**4**) near a single GnRH neuron fiber in the POA (Figure 2-1B, C). Release was observed when the electrode was near the intersection of soma and fibers, or between fibers (both

ME and POA, Figure 2-1B, C), but not near single GnRH fibers even with KCl challenge (Figure 2-1B, C). This provides functional evidence of GnRH release at GnRH-GnRH junctions (7,125).

Differential detection of GnRH release in a model exhibiting diurnal changes in GnRH neuron activity. The above correlation analyses revealed that FSCV accurately detects GnRH release in brain slices. We next tested its ability to measure biologically-relevant changes using a model in which female mice exhibit estradiol-induced daily changes in GnRH neuron activity that are correlated with circulating LH levels (37). This reflects a switch from negative to positive estradiol feedback, and is a critical part of the female reproductive cycle (4,172,173). We hypothesized similar daily changes in GnRH release occur. One-hour FSCV recordings of spontaneous GnRH release were made in the AM (low GnRH neuron activity) and PM (high activity) from both soma-fiber appositions in the POA (AM n=8, PM n=5) and ME (n=6 AM, n=5 PM). No time-of-day differences were observed in the POA for concentration change, release duration or frequency of release (Figure 2-3). Interestingly, 3 of 8 AM POA recordings had no spontaneous GnRH release, but exhibited KCl-evoked release, indicating CFME was placed near a potential release site. The lack of spontaneous release is consistent with reduced activity of GnRH neurons at this time. ME GnRH release was longer in duration ($F(41)=4.292$, $p<0.05$) and exhibited greater concentration changes ($F(41)=3.943$, $p<0.05$) than in the POA regardless of time of the day. Further, release events in the ME were more frequent ($F(26)=5.304$, $p<0.05$) in the PM than in the AM. GnRH concentration changes were greater in the ME during both AM and PM suggesting more release sites than the POA.

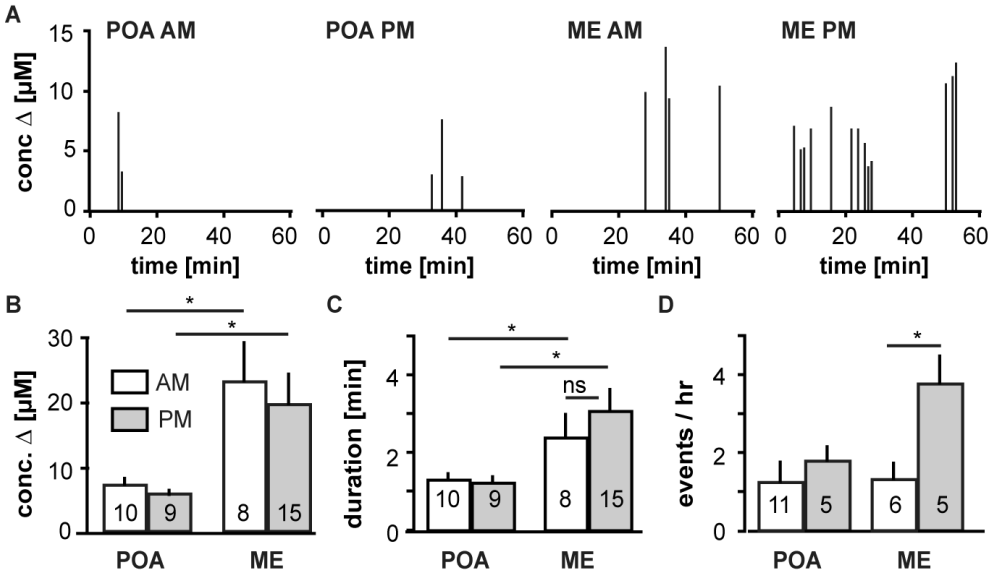

Figure 2-3. . FSCV detects changes in GnRH release between two different biological states. A. Representative examples of the pattern of spontaneous GnRH release detected at CFMEs near GnRH soma/fiber appositions in the preoptic area (POA) and fibers in the median eminence (ME) in ovariectomized mice treated with estradiol to induce diurnal changes in GnRH neuron activity (37). Data were collected in 1-minute bins and each bar shows the maximum concentration in each bin. Multiple spikes in adjacent bin represent one long event. B-D. Mean±SEM concentration change, POA am: n=10, POA pm: n=9, ME am: n=8, ME pm: n=15 (B) event duration, POA am: n=10, POA pm: n=9, ME am: n=8, ME pm: n=15 (C), and number of secretory events/hour, POA am: n=11, POA pm: n=5, ME am: n=6, ME pm: n=5 (D). Numbers in bars are number of cells (D) or number of events examined (B, C); * $p<0.05$.

Discussion

Here we demonstrate that FSCV allows real-time, specific monitoring of GnRH neurosecretion with good spatial resolution in brain sections. FSCV revealed several functional insights into the GnRH network. First, this is the first demonstration in native GnRH neurons that release occurs in regions other than the ME. Second, micromolar changes in GnRH concentration can occur at GnRH-GnRH appositions in the POA and in the ME, demonstrating that GnRH levels that are potentially auto-excitatory are achievable (13). Third, estradiol feedback differentially regulates GnRH release in the POA and ME. Together these data suggest multiple functions of GnRH and potential site-specific regulation of release help sculpt overall output of this neurosecretory network.

In murine brain slices, GnRH release was detected in the POA only where two GnRH neurons appear to interact, indicating that GnRH-GnRH junctions may mark release sites. Although the role of GnRH in the neuroendocrine control of the anterior pituitary is well established, it also acts as a neuromodulator within the brain. This was postulated from *in vivo* studies (157) and such actions have been demonstrated in brain slices (13–15,158). The detection of GnRH release at GnRH-GnRH appositions in the POA provides intriguing evidence that GnRH neurons may utilize GnRH for modulation of their own function. Recent work in cultured embryonic GnRH neurons indicated GnRH was released from the soma and proximal processes (8). The present data support and extend these data by demonstrating that there is at least some degree of anatomical specification of release sites to GnRH-GnRH appositions; further the previous finding of perisomatic release was not attributable to an artifact subsequent to culturing.

FSCV does not allow for detection of absolute concentrations because of the required baseline subtraction; instead, changes in concentration attributable to secretion are monitored. This putative limitation may be an asset in the GnRH neuronal system given the episodic nature of GnRH release. Although absolute concentrations are not determined, the change in concentration measured by FSCV can be used to estimate local concentrations achieved upon release. GnRH release was measured in the micromolar concentration range both at GnRH-GnRH appositions in the POA and within the ME. This high concentration may seem initially surprising given levels measured in pituitary portal blood (5,174), however those levels are quantified post diffusion and dilution in the blood. Further, estimates of synaptic cleft concentrations of transmitters are quite high, for example GABA is in the millimolar range (175). The measurement of micromolar GnRH is of interest with regard to understanding dose-dependent changes in GnRH neuronal activity in response to GnRH (13). Nanomolar exogenous GnRH inhibited GnRH neuron activity, whereas micromolar GnRH increased activity (13). This may be attributable to different affinities of the GnRH receptor when coupled to different G proteins (176). One hypothesis is that random fusion of GnRH vesicles produces low concentrations that inhibit the network, whereas action potential-driven release of GnRH generates high concentrations that excites the system, perhaps contributing to coordination of pulsatile release, or long-term release during the preovulatory GnRH surge (4).

The present work validates FSCV for real-time detection of GnRH in brain slices. The technique is sensitive enough to detect release in the POA between two GnRH neurons, as well as the integrated output of the GnRH population in the ME. Future studies will determine how electrical activity correlates with release from terminals vs. dendrites, and examine the function of GnRH release in different brain regions.

Chapter 3

Differential regulation of gonadotropin-releasing hormone (GnRH) secretion in the preoptic area (POA) and the median eminence (ME) in male mice

Katarzyna M. Glanowska and Suzanne M. Moenter

Abstract

GnRH release in the median eminence (ME) is the central output for control of reproduction. GnRH processes in the preoptic area (POA) also release GnRH. We examined region-specific regulation of GnRH secretion using fast-scan cyclic voltammetry (FSCV) to detect GnRH release in brain slices from adult male mice. Blocking endoplasmic reticulum calcium reuptake to elevate intracellular calcium ($[Ca^{2+}]i$) evokes GnRH release in both the ME and POA. This release is action potential-dependent in the ME but not the POA. Locally-applied kisspeptin induced GnRH secretion in both the ME and POA. Local blockade of IP3-mediated calcium release inhibited kisspeptin-induced GnRH release in the ME but broad blockade was required in the POA. In contrast, kisspeptin-evoked secretion in the POA was blocked by local gonadotropin-inhibitory hormone (GnIH), but broad GnIH application was required in the ME. Although action potentials are required for GnRH release induced by pharmacologically-increased $[Ca^{2+}]_i$ in the ME and kisspeptin-evoked release requires IP3-mediated calcium release, blocking action potentials did not inhibit kisspeptin-induced GnRH release in the ME. Kisspeptin-induced GnRH release was suppressed after blocking both action potentials and plasma membrane Ca^{2+}-channels. This suggests that kisspeptin action in the ME requires both increased $[Ca^{2+}]_i$ and influx from the outside of the cell, but not action potentials. Local interactions among kisspeptin and GnRH processes in the ME could thus stimulate GnRH release without involving perisomatic regions of GnRH neurons. Coupling between action potential generation and hormone release in GnRH neurons is thus likely physiologically labile and may vary with region.

Introduction

Gonadotropin-releasing hormone (GnRH) neurons form the final common pathway for the central control of reproduction. This is accomplished via episodic secretion of GnRH near portal vessels in the median eminence (ME) to regulate the anterior pituitary (3,5). In addition to this well-established neuroendocrine output, recent evidence indicates GnRH is also released in other brain regions, specifically in the preoptic area (POA) (10). This latter observation is consistent with a recent report of perisomatic release in cultured primate GnRH neurons (8), and with reports that GnRH has central neuromodulatory roles in addition to neuroendocrine function (13–15,157,158). Central GnRH has been postulated to take part in direct synchronizing of the GnRH neuron network, in local circuit feedback on GnRH neurons and in altering sex behavior (146,177,178).

Peptide release has also been observed from the dendrites/soma of magnocellular neuroendocrine neurons located in the supraoptic nucleus. The neuroendocrine function of these neurons is to release vasopressin and oxytocin from terminals in the posterior pituitary, (130). Interestingly, this release appears to be regulated independently of secretion to the blood and to utilize different mechanisms. Specifically, large dense core vesicles containing peptides can be released in an action potential-independent manner that requires increased intracellular Ca^{2+} levels, and can be initiated by signaling arising from ligand interactions with metabotropic receptors (179–182). Parvocellular neuroendocrine neurons, including GnRH neurons, have not been studied in this regard, but many neuromodulators have been reported to regulate GnRH release (183). Of these, kisspeptin is among the most potent activators and gonadotropin-inhibitory hormone (GnIH) is among the most potent inhibitors. GnRH neurons express the kisspeptin receptor kiss1R and are strongly depolarized by kisspeptin (184,185).

Kisspeptin administration increases GnRH release, and thereby increases secretion of the pituitary gonadotropin luteinizing hormone (LH) (186). GnIH can act via GPR147, which is expressed centrally as well as in the pituitary. In brain slices, GnIH inhibits GnRH neuron action potential firing, even in the presence of kisspeptin (187,188), suggesting the interaction of these two neuromodulators is critical to the overall activity of GnRH neurons.

The diffuse distribution of GnRH neurons and distance from the soma to neuroendocrine terminals favors the study of region-specific regulation of release, but a sufficiently sensitive method that can be visually targeted to specific sites has not been available. We adapted an electrochemical method, fast scan cyclic voltammetry (FSCV), for GnRH detection directly in mouse brain slices (10). Using FSCV, both POA and ME release can be detected, allowing studies of the mechanisms underlying release in these regions. Here we utilize FSCV to investigate action potential dependence, calcium dependence and neuropeptidergic (kisspeptin and GnIH) regulation of GnRH secretion in the ME and POA.

Materials and Methods

Animals Adult 40-90 day-old gonad-intact male GnRH-eGFP mice (6) and kisspeptin knock-out mice (189) (generous gift of Drs. Yee-Ming Chan and Stephanie Seminara, Massachusetts General Hospital) were housed under a 14:10 h light:dark photoperiod (lights on at 0300 EST) with Harlan 2916 chow and water available ad libitum. All procedures were approved by the University of Michigan University Committee on the Use and Care of Animals.

Brain slice preparation All chemicals were purchased from Sigma Chemical Company (St Louis, MO) unless noted. Mice were decapitated and brain slices were prepared as

described (9,67). All buffers were bubbled with 95%O2/5%CO2 15min before usage. Sagittal 300 μm brain slices were cut using a Leica VT 1200S vibratome (Leica Biosystems, Buffalo Grove, IL) in ice-cold sucrose saline containing (in mM): 250 sucrose, 3.5 KCl, 26 NaHCO3, 10 glucose, 1.25 NaH2PO4, 1.2 MgSO4, and 3.8 MgCl2. Slices were incubated 30min at room temperature in a 1:1 mixture of sucrose saline and artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2.5 CaCl2, 1.2 MgSO4, and 10 D-glucose, pH 7.4, then transferred to 100% ACSF and incubated 30 –300min at room temperature before study.

Fast-scan cyclic voltammetry (FSCV) Individual slices were transferred to a recording chamber mounted on the stage of an upright microscope (Olympus BX50WI; Opelco, Dulles, VA). The chamber was perfused continuously with ACSF at a rate of 5–6ml/min at 31–32°C. Slices were stabilized in the chamber for at least 10min before recording. GnRH detection was monitored using carbon fiber microelectrodes (CFMs) manufactured as previously described (163). FSCV recordings were made in voltage-clamp mode of an EPC-10 USB amplifier running Patchmaster (HEKA Elektronik, Lambrecht/Pfalz, Germany) on a Macintosh Mac Pro computer (Apple Computers, Cupertino, CA). Electrode potential was continuously scanned from 0.5 to 1.45 V at 400V/s every 100ms. GnRH-GFP neurons were identified by brief illumination at 470nm. CFMs were placed either among GnRH neuron terminals in the ME or near GnRH fiber-fiber appositions in the POA (Figure 3-1).

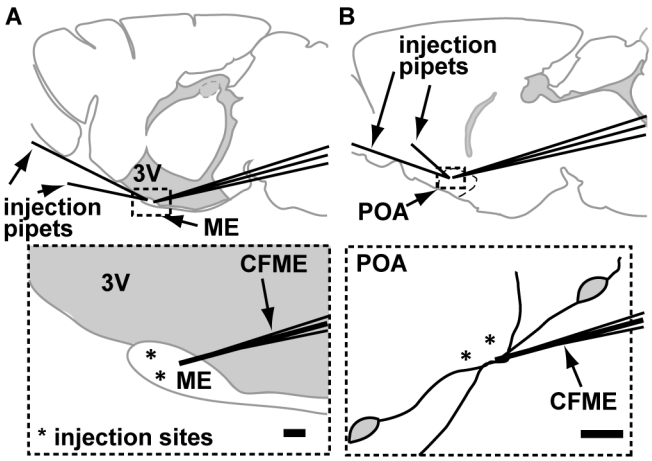

Figure 3-1 Illustration of experimental setup with relative positions of electrode for FSCV and injection sites in the ME (A) and POA (B). Upper panels show sagittal slice with relative positions of the carbon fiber microelectrode (CFME) for FSCV and injection pipets for local drug application. CFME and pipets are separated by 20-30μm. 3V- third ventricle. Dotted areas are expanded in the lower panels, asterisks in lower panel indicate injection sites.

Experimental design The general design of experiments was as follows. The voltage protocol for GnRH detection was run for 10-15min after CFMs were placed in the tissue before collecting data to allow recordings to stabilize. Release was recorded continuously for the duration of each experiment, during which a variety of treatments were applied by brief (10-20sec) local injection via a pipette placed 20-30μm from the CFM or by bath treatment. Intervals and timing of these treatments are specified for each study below. At the end of each recording the viability of slice was confirmed by its ability to release GnRH in response to 20mM KCl applied 20min after the final experimental treatment. Only slices that released GnRH in response to KCl are included in the analysis.

First, we investigated the Ca^{2+}- and action potential dependence of GnRH secretion in ME vs. POA. Local injection of thapsigargin (5μM, Tocris Biosciences, Bristol, UK) or

cyclopiazonic acid (10μM CPA) was used to elevate cytoplasmic calcium levels (190,191). To test if multiple releases could be induced, thapsigargin or CPA was injected at 20min intervals. To test the requirement for action potential generation, thapsigargin was injected under control conditions, then the slice was bathed with 0.5μM tetrodotoxin (TTX, Calbiochem, Billerica, MA) for 5 to 10min to block sodium channel-dependent action potentials and release in response to thapsigargin injection tested again. To test the requirement for kisspeptin release, the ability of CPA to induce GnRH release in the ME was tested in kisspeptin KO mice. POA studies were not done in kisspeptin KO mice as GFP is required to target the CFMs in the POA and GFP is not expressed in GnRH neurons this mouse line.

Next, to test effects of neuromodulators on GnRH release from the POA and ME, 10nM kisspeptin (Phoenix Pharmaceuticals, Burlingame, CA) alone or in combination with 1μM GnIH (Phoenix) was locally injected into the slice. Kisspeptin alone was similarly injected during bath application of 1μM GnIH. The interval between treatments was 10-15minutes.

Next, we investigated the role of intracellular Ca^{2+} mobilization in kisspeptin-evoked GnRH release in the POA and ME we blocked IP3-mediated release of Ca^{2+} from the endoplasmic reticulum with xestospongin C (XC, 20μM Tocris). Kisspeptin was injected locally three times at 10-15min intervals: under control conditions (kisspeptin only), with local injection of XC, and after washout of XC. In some studies in the POA, kisspeptin was locally injected under control conditions and during bath application of XC (5μM).

To determine if kisspeptin-evoked GnRH release in the ME was action potential dependent, three local kisspeptin injections were performed at 10-15min intervals: under control conditions, then in the presence of bath-applied TTX (0.5μM, 3-4min before

second kisspeptin injection), and then in the presence of both TTX and the calcium channel blocker Cd^{2+} (200μM 3-4min before third kisspeptin injection).

Analysis FSCV data were converted to general text format using Igor Pro (Wavemetrics, Lake Oswego, OR) then analyzed using Demon software (generously provided by Jordan Yorgason, Rodrigo Espana and Sara Jones, Wake Forest University Health Sciences) as described previously (10,163). Cyclic voltammograms (CVs) were background-subtracted by averaging 10 background scans. To verify the identity of a spontaneous release peak as GnRH, five control CVs collected after GnRH was injected into a slice were averaged. Each putative GnRH CV was correlated with this average and was considered to be GnRH if R2 ≥0.8. This threshold was set to allow for electrode variability. 92% of CVs passed this test. Changes in GnRH concentration were estimated based on CFM calibration in 5μM GnRH.

Data were transferred to a Prism5 for statistical analysis (GraphPad Software, San Diego, CA). Parameters measured included release event duration, GnRH concentration change, lag to GnRH release after each pharmacological treatment and age of treatments resulting in evoked GnRH secretion. Data were analyzed by parametric or non-parametric ANOVA tests as dictated by data distribution followed by two-tailed post-hoc analyses. Data are presented as mean±SEM and $p<0.05$ was considered significant.

Results

Intracellular Ca^{2+}-evoked GnRH release is action potential-dependent only in the ME. Mechanisms leading to neurosecretion can differ between dendritic/somatic and terminal regions (132). We used brief exposure to 5μM thapsigargin injected locally into the ME or POA as illustrated in Figure 3-1A, B, to elicit release. Thapsigargin blocks Ca^{2+} reuptake into the endoplasmic reticulum by inhibiting SERCA pumps, and brief applications as used here tend to increase intracellular calcium (179,192,193). Thapsigargin was first injected under control conditions and then after blocking sodium-dependent action potentials with tetrodotoxin (0.5μM, timeline Figure 3-2A).

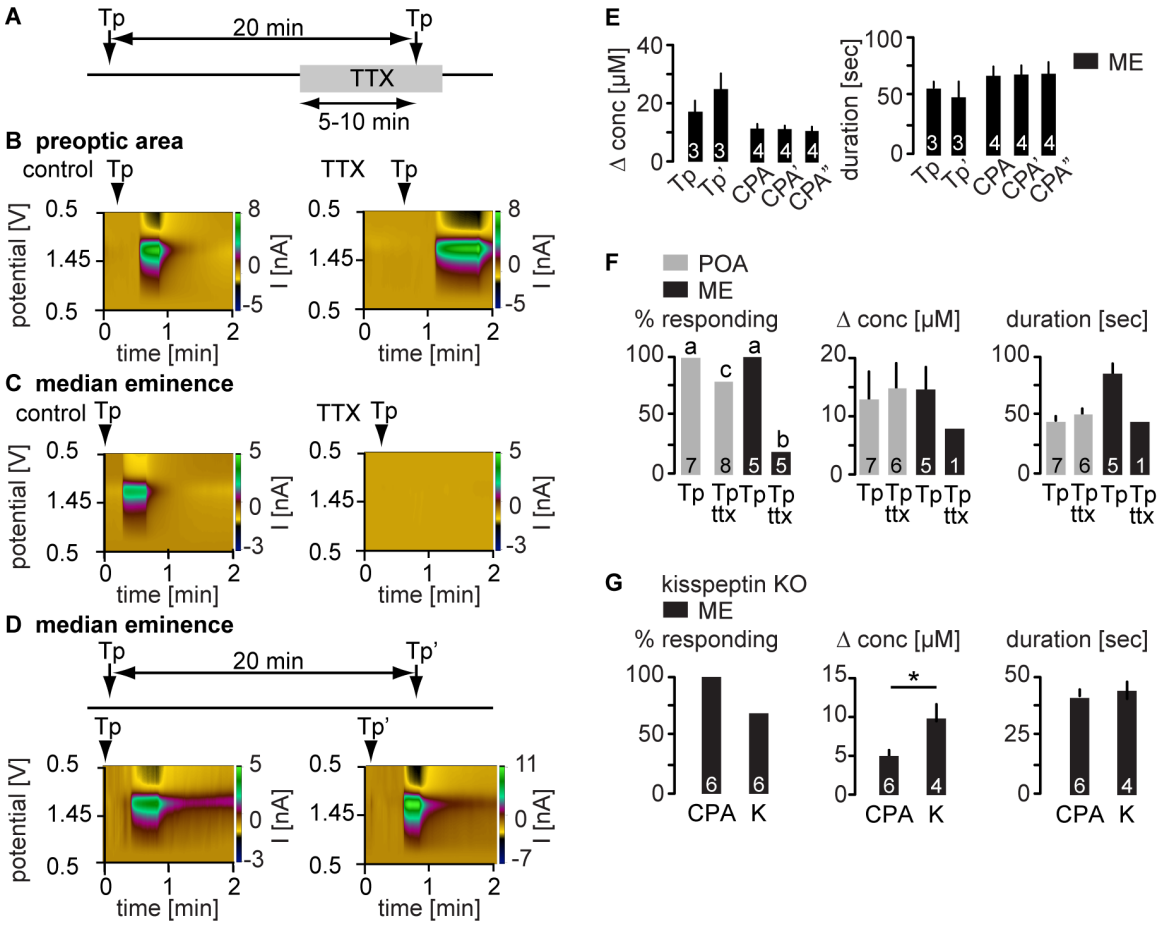

Figure 3-2 Increasing intracellular Ca^{2+} by blocking SERCA pumps elicits GnRH release in the POA and ME, but requires action potentials only in the ME. A. Experimental timeline for B, C. B,C. Representative examples of thapsigargin (Tp)-evoked GnRH release shown as a current heat map during the control period (left) and TTX treatment (right) in the POA (B) and ME (C). D. Top, experimental timeline. Bottom, repeated injection of Tp under control conditions induces repeated GnRH release in the ME. E. Mean±SEM peak GnRH release amplitude (left) shown as change in concentration, and release duration (right). Numbers within bars indicate sample size. F. Mean±SEM percent responding to Tp (left), concentration change (center), and release duration (right). Grey bars show POA, black bars show ME. Lower case letters indicate $p<0.05$ Chi square test. G. GnRH release in response to CPA is not blocked in kisspeptin (K) KO mice. Mean±SEM percent responding to CPA or kisspeptin (K) (left), concentration change (center), and release duration (right), asterisks indicate $p<0.05$ with Student's t-test.

Thapsigargin reliably induced GnRH release in both the POA (n=7 of 7 trials, Figure 3-2B, F) and ME (n=5 of 5 trials, Figure 3-2C, F) under control conditions. Thapsigargin-induced release persisted in the POA with similar magnitude and duration after treating the slice with TTX to block action potentials. (Figure 3-2B, F), In marked contrast, blocking action potentials inhibited thapsigargin-induced GnRH release in the ME (Figure 3-2C, F).

To test if the lack of response to a second application of thapsigargin in the ME was due to inability of thapsigargin to elicit repeated releases, thapsigargin (Figure 3-2D, E) or CPA, a drug with the same mechanism of action (Figure 3-2E) were repeatedly injected under control conditions (i.e., no TTX). Both thapsigargin (n=3) and CPA (n=4) elicited repeated GnRH release in 100% of trials in the ME with no decrement in either amplitude or duration. Neither GnRH concentration change nor duration in response to thapsigargin differed between regions (Figure 3-2F). The requirement for action potentials for intracellular calcium elevations induced by blocking endoplasmic reticulum reuptake to induce GnRH release suggested the possibility that Ca^{2+} was elevated in a cell upstream of the GnRH neuron itself and that upstream cell needed to generate action potentials to release a subsequent mediator to induce GnRH release. To test the hypothesis that this intermediate mediator was kisspeptin, the ability of CPA to induce GnRH release in the ME was tested in kisspeptin KO mice (in the absence of TTX). CPA repeatedly induced GnRH release in kisspeptin knockout mice in the median eminence (Figure 3-2G), although GnRH concentration change in response to CPA was lower than that in response to kisspeptin (Figure 3-2G). All together these data suggest that GnRH secretion in response to increased intracellular Ca^{2+} can be action potential-independent in the POA.

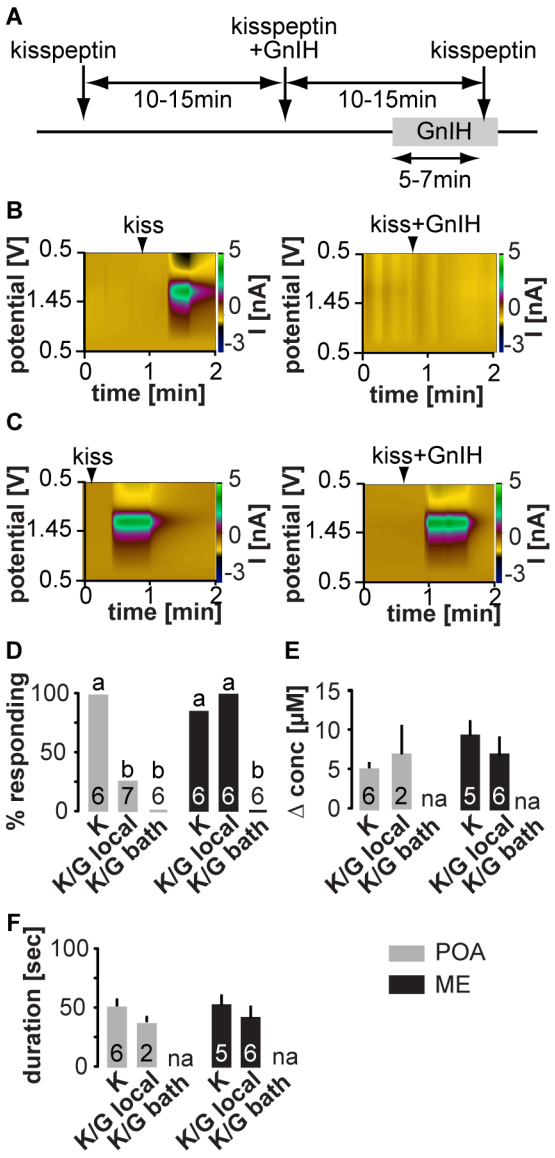

Figure 3-3 Neuropeptide modulation of GnRH secretion in the ME and POA. A. Experimental timeline arrows indicate local, grey bars indicate bath application. B, C. Representative examples of local kisspeptin-evoked GnRH secretion in the POA (B) and ME (C). Left panel shows response to local injection of kisspeptin alone in each region, right panel shows response to local injection of both kisspeptin and GnIH (i.e., first two injection arrows in time line). D-F. Mean±SEM percent of cells responding to kisspeptin (D), peak GnRH release amplitude (E) shown as change in concentration, and release duration (F). Grey bars show POA, black bars show ME. Lower case letters indicate $p<0.05$ Chi square test.

Local GnIH blocks kisspeptin-induced GnRH release in the POA, but bath GnIH application is required in the ME. We next examined the effects of two established modulators of GnRH neuron action potential firing, kisspeptin and GnIH, on GnRH release (184,187). GnRH release in response to 10nM kisspeptin was monitored in both the ME and POA when given alone, then after local injection of 1μM GnIH, and finally after bath application of GnIH (Figure 3-3A). To minimize the effects of kisspeptin on the neuronal network upstream of GnRH neurons, kisspeptin was always applied locally through brief injection directly to the slice. In both the POA (n=7 of 7) and ME (n=6 of 7), kisspeptin potently and repeatedly induced GnRH secretion (Figure 3-3). There were no differences in either event duration or concentration change between regions or with repeated application (Figure 3-3D-F). In the POA, local co-application of GnIH blocked GnRH release in 5 of 7 trials. In contrast local GnIH failed to block kisspeptin-induced GnRH release in all 6 trials in the ME. This suggested either that GnIH receptors were not near the application site in the ME or that GnIH is ineffective in blocking GnRH release in this preparation. To test if widespread application of GnIH could block GnRH release induced by local kisspeptin injection, GnIH was bath applied. Bath-applied GnIH completely blocked kisspeptin-evoked GnRH secretion in both brain regions (n=6 each, Figure 3-3D-F).

Kisspeptin-evoked GnRH secretion requires both mobilization of intracellular Ca^{2+} stores and extracellular Ca^{2+} influx. In the next set of experiments, we investigated the intersection between neuromodulators and intracellular calcium mobilization. Kisspeptin acts via a Gq-coupled receptor, depleting PIP2 and activating DAG and IP3 production (194); any of these mechanisms might alter GnRH release. To test the hypothesis that kisspeptin-induced GnRH secretion requires release of Ca^{2+} from intracellular stores, we used XC to block IP3-dependent Ca^{2+} release (Figure 3-4A). Local administration of

20μM XC largely prevented kisspeptin from eliciting GnRH secretion in the ME (release observed in 1 of 6 cases) but not POA (release observed in 5 of 5 cases, Figure 3-4). To test the possibility that XC targets in the POA are distal to kisspeptin application site, XC (5μM) was bath applied. Bath-applied XC fully blocked kisspeptin-induced GnRH secretion in all 7 slices tested (Figure 3-4F-H). These results suggest that action of intracellular Ca^{2+} mobilization in kisspeptin-evoked GnRH secretion is required in both the ME and POA. Moreover, the spatial distribution of XC targets (most likely endoplasmic reticulum), kisspeptin receptors and/or their downstream mediators, and GnRH release sites differ between the POA and ME.

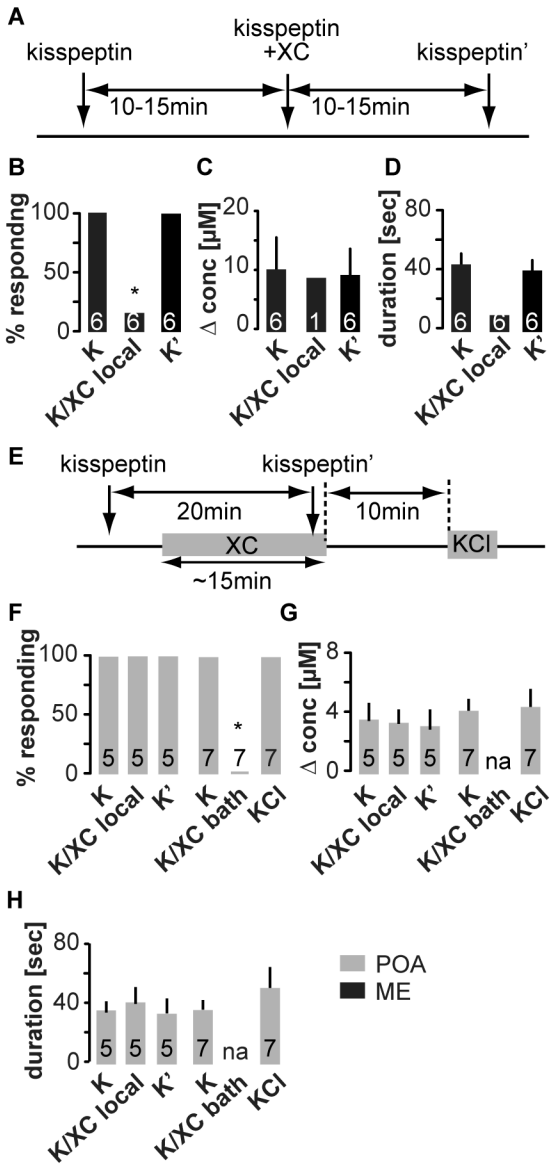

Figure 3-4 Pharmacological blockade of IP3-dependent intracellular Ca^{2+} release reduces kisspeptin-induced GnRH secretion. A. Experimental timeline for B-D; arrows indicate local, grey bars indicate bath application. B-D. Mean±SEM percent of cells responding to kisspeptin in the median eminence (D), peak GnRH release amplitude (E) shown as change in concentration, and release duration (F). Numbers within bars indicate sample size; asterisks indicate $p<0.05$ Chi square test. E. Experimental timeline for F-H; arrows indicate local, grey bars indicate bath application. F-H. Mean±SEM percent of cells responding to kisspeptin in the POA (D), peak GnRH release amplitude (E) shown as change in concentration, and release duration (F). Numbers within bars indicate sample size; asterisks indicate $p<0.05$ Chi square test.

Finally, we investigated if kisspeptin-induced release requires generation of action potentials. This was tested only in the ME because release induced by increasing intracellular Ca^{2+} was only action-potential dependent in this region. Kisspeptin (10nM) was locally injected into the ME before and during treatment with TTX (Figure 3-5A). In 10 of 10 slices kisspeptin still evoked GnRH secretion when action potentials were blocked (Figure 3-5B-D). To test if blockade of cell membrane voltage-dependent Ca^{2+} channels in addition to action potentials inhibits kisspeptin-induced GnRH release, we repeated the experiment in the presence of 200μM Cd^{2+}. This treatment blocked kisspeptin induction of GnRH secretion in the ME (n=5 of 5, Figure 3-5B-D), suggesting that Ca^{2+} from both intracellular stores and outside of the cell underlies kisspeptin-induced GnRH secretion.

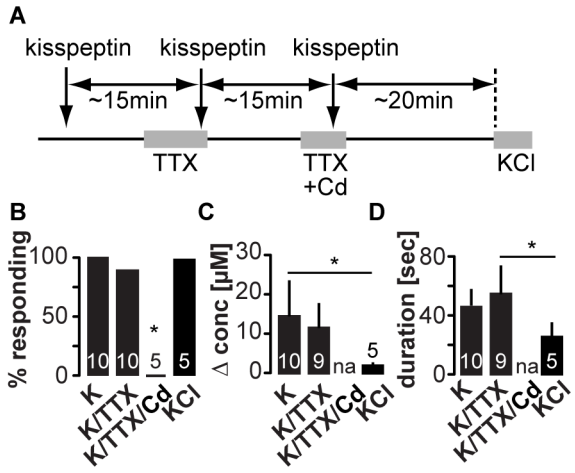

Figure 3-5 Blockade of both action potentials and soma membrane voltage-gated calcium channels prevents kisspeptin-induced GnRH release in the ME. A. Experimental timeline; arrows indicate local, grey bars indicate bath application. B-D. Mean±SEM percent of cells responding to kisspeptin in the median eminence (D), peak GnRH release amplitude (E) shown as change in concentration, and release duration (F). Numbers within bars indicate sample size; asterisks indicate $p<0.05$ Chi square test (B), One-way ANOVA with Tukey's multiple comparison test (C, D).

Discussion

Pulsatile GnRH secretion from the ME into the pituitary portal vasculature is a crucial driver of reproductive function and local release of GnRH in the POA may serve a variety of neuromodulatory roles. Little is known, however, about the mechanisms underlying release in the ME vs. POA. Here we used a FSCV, a localized detector of GnRH release (10,195), in combination with localized delivery of neuromodulators and/or agents to alter directly intracellular signaling. We demonstrate both similarities and differences in the region-specific regulation of GnRH release that may be attributable to relative location of cell surface receptors and GnRH release sites, as well as to signaling to and within the GnRH neurons.

Electron microscopic studies indicate that GnRH is localized to large dense-core vesicles in the soma, dendrites, axons and terminals of GnRH neurons (117,119,196,197). We hypothesized that, consistent with other neurosecretory systems, increased intracellular Ca^{2+} is necessary for the release of those vesicles, and tested this by brief local injection of thapsigargin or CPA to block Ca^{2+} reuptake into the endoplasmic reticulum. Thapsigargin has been reported to rapidly increase intracellular calcium levels in immortalized GnRH neurons (68,71). Blocking SERCA pumps to elevate intracellular Ca^{2+} rapidly and repeatedly evoked GnRH release in both the POA and ME. Since treatments were delivered near the site of GnRH measurement, their effective zone is spatially limited. It is not possible, however, to determine if treatments act directly upon GnRH neurons, on their afferents, on local glia or a combination of these. Signals between neurons would likely require action potentials in the presynaptic cell to release an intermediate signal, thus blocking action potential firing may reveal clues as to the site of action. Further, thapsigargin was reported to increase action potential firing in immortalized GnRH neurons (71). We thus determined if SERCA

blocker-induced GnRH release required action potentials. In the POA, GnRH release was action potential-independent; this suggests that GnRH release is most likely evoked by SERCA inhibition directly in GnRH neurons in the somato-dendritic region of these cells. Action potential-independent modification of neuromodulator release from presynaptic neurons cannot, however, be completely excluded (198).

In contrast to the POA, action potentials were required for thapsigargin-induced GnRH release in the ME. This indicates that propagation of a signal is needed either between an afferent neuron and the GnRH neuron, or to connect more distant elements within the GnRH neuron itself (in the case of SERCA blockers, endoplasmic reticulum and GnRH release sites). With regard to signaling from an afferent neuron, CPA-evoked GnRH release persisted in the ME of kisspeptin KO mice. Thus the present observation that blocking action potentials blocks the ability of elevating intracellular calcium with SERCA blockers to induce GnRH release is not attributable to a failure of kisspeptin release from afferent neurons. This does not eliminate the possibility that SERCA blockers are increasing action potential firing and subsequent neurosecretion from an afferent neuron. In this regard, activation of neurokinin 3 receptors also induces GnRH release in the ME of kisspeptin KO mice (195). Neurokinin 3 receptors are the high affinity binding site for neurokinin B (199), a peptide that is coexpressed in some kisspeptin neurons (200) and shown to be important for fertility (201). Further, tanycytes surrounding GnRH terminals contain endoplasmic reticulum (117,119). Thus indirect actions via either neurons or glia are possible explanations for the action potential dependence of SERCA-blocker induced GnRH release in the ME. These data support and extend previous reports of differences between central/dendritic and peripheral/terminal release in the magnocellular neuroendocrine system releasing oxytocic and vasopressin. Dendritic release of these peptides can be elicited independent of action potential firing, whereas

secretion from axon terminals is typically activity-dependent (198). Dendritic release involves mobilization of Ca^{2+} from thapsigargin-sensitive stores for both oxytocin and vasopressin (181), and further calcium influx due to activation of voltage-sensitive Ca^{2+} channels for vasopressin (181,182). For the GnRH system, coordination possibly provided by action potential-driven release in the ME may be critical for generating the pulsatile pattern required by the pituitary.

The physiological activator of increased intracellular calcium required for dendritic release in magnocellular neurons is proposed to be ligand-receptor interactions (132). In GnRH neurons, kisspeptin is a potent stimulator of action potential firing (184) and hormone release into the pituitary portal vasculature via the ME (202). The kisspeptin receptor (kiss1r) is coupled to Gq and signals via PIP2 depletion and IP3 (203,204). We demonstrate here that GnRH secretion can be evoked by kisspeptin in an IP3-dependent manner not only in the ME (205) but also in the POA. In the POA, however, it was necessary to block IP3-mediated calcium release over a wider area to inhibit kisspeptin-induced GnRH release. This suggests the kisspeptin receptor and intracellular Ca^{2+} stores are further apart in the GnRH neuron processes in the perisomatic region than they are in the GnRH neuron terminals in the ME. Further, the localized nature of both kisspeptin application and GnRH measurement used in the present study indicates that, in both the POA and ME, kisspeptin receptors and sites of GnRH release are likely overlapping (Figure 3-6). Interestingly, local injection of kisspeptin repeatedly induced GnRH release with no decrement under the conditions used in the present studies. This is in contrast to reports of desensitization of GnRH neuron action potential firing with repeated bath application of kisspeptin, even when bath application was a single drop to the slice recording chamber with a high flow rate (40,206). Further, the action potential firing increases in those studies lasted several

minutes (typically >20 min), compared to relatively brief pulses of release in the present study lasting under two minutes. This may be due to a lower concentration and smaller volume and therefore smaller mass of kisspeptin being delivered in a more localized manner in the present study. While clearly effective in eliciting GnRH release, the amount of kisspeptin used in the present study may not induce desensitization and also may be more easily washed away. With regard to the latter, studies of kisspeptin signaling in cultured cells indicate that intracellular elevations in Ca^{2+} are prolonged unless kisspeptin is removed from the medium (207). It is also possible that the bath application methods used in the previous studies more broadly engaged additional parts of the upstream network, thereby resulting in prolonged stimulation of GnRH neuron activity. Since GnRH neurons express kiss1R, kisspeptin action is likely at least partially directly on GnRH neurons. Kisspeptin can, however, also excite GnRH neurons indirectly by increasing excitatory GABAergic as well as glutamatergic transmission to these cells (40,86,88,208).

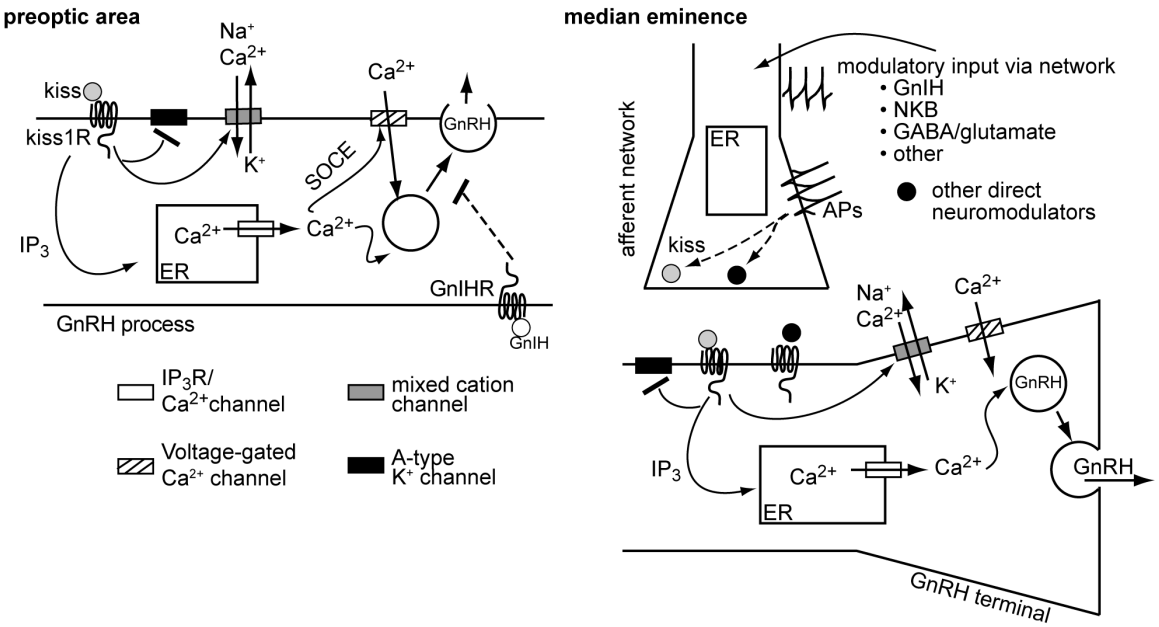

Figure 3-6 Possible models for the differential regulation of GnRH release in the preoptic area and median eminence. In the POA (left), GnRH release can occur without action potentials and can be locally inhibited by GnIH. We postulate all the elements necessary for control may be contained locally within the GnRH process itself. It is important to point out that influences from afferent neurons are not excluded. For example, kisspeptin clearly influences release, likely via direct action on the neuronal process. In the ME (right), while kisspeptin can induce GnRH release in an action-potential independent manner, suggesting the GnRH neuron itself does not need to generate spikes for secretion, regulation of GnRH release also appeared to depend more on the upstream network because 1- elevating intracellular Ca^{2+} by blocking endoplasmic reticulum (ER) reuptake was unable to induce GnRH release in the absence of action potentials, 2-GnIH did not inhibit GnRH release locally, suggesting it is sculpting secretory pattern via the network, and 3-it is likely that endogenous release of kisspeptin and other neuromodulators is action potential-dependent. We hypothesize that by injecting kisspeptin locally the requirement for GnRH neuron action potential firing was bypassed and kisspeptin directly stimulated GnRH release from the GnRH terminal. SOCE, store operated calcium entry.

The difference in duration of kisspeptin-induced firing and kisspeptin-induced release suggests the postulate that the robust and prolonged increase in GnRH neuron firing rate in response to kisspeptin may not generate prolonged neurosecretion. Of further interest in this regard, blocking action potential firing did not prevent kisspeptin from eliciting GnRH secretion in the median eminence without concomitant blockade of Ca^{2+} influx via Cd^{2+}-sensitive channels. The apparent discrepancy between action potential dependence of GnRH release evoked by thapsigargin and action potential-independent release evoked by kisspeptin in the median eminence can be explained in a couple of ways. First, IP3-mediated release of calcium subsequent to kiss1r activation would not be blocked by preventing action potential firing if kisspeptin were acting on kiss1r expressed by GnRH neurons, providing further evidence for direct action of kisspeptin on GnRH neurons to evoke GnRH release. Second, kisspeptin activates mechanisms in addition to intracellular calcium mobilization in GnRH neurons, including reducing A-type potassium currents (209) and increasing TRPC4 currents (204,210) and non-specific cation currents (211). Third, depletion of PIP2 from the plasma membrane can modulate a variety of ion channel activities (212–217) that could ultimately change secretory output.

Another neuropeptide involved in control of GnRH neuron activity is GnIH. It inhibits both electrical activity of GnRH neurons (187,218) and secretion of gonadotropic hormones from the pituitary (219). GnIH was also recently implicated in prepubertal suppression of LH levels (220,221). These findings suggest one mechanism underlying GnIH inhibition of LH secretion may be via suppression of GnRH release. We demonstrate that GnIH counteracts the stimulatory effect of kisspeptin on GnRH release in both the POA and ME. In contrast to the tight overlap of kisspeptin receptors and GnRH release, however, there were region-specific differences in spatial distribution of GnIH targets. In the ME,

local co-application of GnIH with kisspeptin did not prevent kisspeptin-induced GnRH secretion, suggesting no or insufficient activation of GnIH receptors near kisspeptin receptors in this region (Figure 3-6). Consistent with this observation, immunohistochemistry has demonstrated little to no GnIH immunopositive fibers in the ME (222–224). This suggests GnIH receptors may not be present in the ME. In the POA, however, local GnIH effectively prevented kisspeptin-evoked GnRH release in the majority of cases, suggesting more proximal location of kisspeptin receptors and GnIH receptors in this region (Figure 3-6). Consistent with this observation, some GnRH neurons have been reported to express GnIH receptors (225). This observation contrasts somewhat with previous electrophysiological studies demonstrating that GnIH inhibits firing only in subset of GnRH neurons (187,218). GnIH could block GnRH release via mechanisms not involving firing inhibition, another possible indication of uncoupling between GnRH neuron action potential generation and GnRH release. Moreover, bath application of GnIH suppressed kisspeptin-evoked release in both POA and ME. This suggests GnIH may prevent kisspeptin-induced release of GnRH via upstream networks.

Location-dependent regulation of GnRH release in the POA vs. ME suggests regional differences in GnRH neuron processes, particularly in the arrangement of neuromodulator receptors, intracellular signaling systems and GnRH release sites in these two regions (Figure 3-6). The limited electrophysiological studies performed in distal GnRH processes approaching the ME suggest these processes receive fast synaptic transmission (226). A similar arrangement is likely for neuromodulatory inputs, and is indeed suggested by the differential regulation of GnRH release observed in the present study. Such distinct regulatory mechanisms allow GnRH release in each region to be independent of the other. This facilitates the fine-tuning of the GnRH output to

serve a variety of different physiological functions both within the brain and exiting the brain as neuroendocrine output to the pituitary.

Chapter 4

Development of gonadotropin-releasing hormone secretion and pituitary response

Katarzyna M. Glanowska, Laura L. Burger and Suzanne M. Moenter

Abstract

Acquisition of a mature pattern of gonadotropin-releasing hormone (GnRH) secretion from the central nervous system is a hallmark of the pubertal process. Little is known about GnRH release during sexual maturation, but it is assumed to be minimal before later stages of puberty. We studied spontaneous GnRH secretion in brain slices from male mice during perinatal and postnatal development using fast scan cyclic voltammetry (FSCV) to detect directly the oxidation of secreted GnRH. There was good correspondence between the frequency of GnRH release detected by FSCV in the median eminence of slices from adults with previous reports of in vivo LH pulse frequency. The frequency of GnRH release in the late embryonic stage is surprisingly high, reaching a maximum in newborns and remaining elevated in one-week-old animals despite low luteinizing hormone (LH) levels. Early high-frequency GnRH release was similar in wild type and kisspeptin knockout mice indicating this release is independent of kisspeptin-mediated excitation. In vivo treatment with testosterone or in vitro treatment with gonadotropin-inhibitory hormone (GnIH) reduced GnRH release frequency in slices from 1wk old mice. The GnIH antagonist RF9 restored GnRH release in slices from testosterone-treated mice, suggesting testosterone inhibition is GnIH-dependent. At 2-3wk of age, GnRH release is suppressed before attaining adult patterns. Reduction in early life spontaneous GnRH release frequency coincides with the onset of the ability of exogenous GnRH to induce pituitary LH secretion. These findings suggest lack of pituitary secretory response, not lack of GnRH release, initially blocks downstream activation of the reproductive system.

Introduction

Puberty leads to full sexual maturation and involves both physiological and behavioral changes in vertebrates. This complex process occurs on multiple levels including gene expression, epigenetic modifications, and functional reorganization of neuronal and glial networks in the brain regions controlling reproduction (143,227–230). From a neuroendocrine perspective, in most species the pubertal process is thought to culminate in the activation of the GnRH neural network (231,232); in primates this network appears to be reactivated at puberty following a neonatal elevation in pituitary gonadotropin levels (and presumably GnRH release) that is subsequently suppressed during the juvenile period (152). In other species, a similar prolonged increase in gonadotropins during early postnatal life does not occur. Rather brief increases in LH appear primarily during the immediate perinatal period (233) In mice, this is confined to males, only observed during the first 4h post birth, and not dependent upon GnRH (153). LH levels in male mice remain low until a mild increase about 12-15d post partum (154,155), followed by an increase to sexual maturity when pulses of LH are detected every 2-3h (148).

In adults, GnRH pulses released from the median eminence (ME) into pituitary portal blood strongly correlate with LH pulses in peripheral circulation (5). This strong correlation in adulthood has lead to the logical assumption that low levels of gonadotropins typically observed during first days to weeks of life reflect relative inactivity of GnRH release, although sporadic increases may indicate infrequent GnRH secretion (147,154,155,234,235). The subsequent persistent rise in LH levels during later development is considered to mark the onset of GnRH pulse generator activity. Consistent with this postulate, the frequency of GnRH release from isolated rat retrochiasmatic brain explants increased from d12 to d27 (149), as did *in vivo* GnRH

release frequency in late pubertal monkeys and rats (236,237), although it is important to point out that LH was not monitored during these studies, nor were earlier ages examined.

The understanding of development of GnRH release that emerges from the above studies is hampered by the need to interpolate dynamic changes in GnRH release from single samples measuring downstream gonadotropin levels; these samples are often terminal and/or pooled among separate subjects. At present, there are no direct measurements of the pattern GnRH release from the embryonic stage through completion of puberty in any species. Here we used fast scan cyclic voltammetry (FSCV), an electrochemical method that can directly quantify changes in GnRH release (10), to examine the developmental profile of GnRH release in the median eminence and to study its regulation.

Materials and Methods

Animals and brain slice preparation. GnRH-eGFP (6) or kisspeptin knockout (189,238) mice were housed under a 14:10h light:dark photoperiod with Harlan 2916 chow and water available *ad libitum*. On the day of study, brain slices were prepared as described (9,67). All chemicals were obtained from Sigma Chemical Company unless noted. All buffers were bubbled with 95%O_2/5%CO_2 15min before use. Brain slices (300-350μm) were cut using a Leica VT 1200S Vibratome 3000 in ice-cold sucrose saline containing the following (in mM): 250 sucrose, 3.5 KCl, 26 NaHCO3, 10 glucose, 1.25 NaH2PO4, 1.2 MgSO4, and 3.8 MgCl2. Slices were incubated at room temperature for 30min in a 1:1 mixture of sucrose saline and artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5

$CaCl_2$, 1.2 $MgSO_4$, and 10 D-glucose, pH7.4, then transferred to 100%ACSF and incubated at room temperature 30-300min before study.

FSCV recordings. Individual brain slices containing the median eminence, which is where neuroendocrine release of GnRH to control the pituitary gland occurs, were transferred to a recording chamber mounted on the stage of an upright microscope (Olympus BX50WI). The chamber was perfused with ACSF at a rate of 5–6ml/min at 31–32C. Slices were stabilized in the chamber for >10min before recording. GnRH release was detected using carbon fiber microelectrodes (10). Microelectrodes were placed among GnRH fibers in the median eminence and stabilized for 15min before collecting data. Potential was continuously scanned from 0.5 to 1.45V at 400 V/s every 100ms (Figure 4-1A). Signals arising from spontaneous release were recorded for 2-4h in each individual slice. Release events separated by <1min were counted as a single event for analysis, as the pituitary is unlikely to distinguish such events *in vivo*. Such short intervals were observed only at E18, P1 and 1wk; the values reported for these ages are thus a conservative estimate of release frequency.

To test the response to kisspeptin, kisspeptin 10 (10μM Phoenix Pharmaceuticals) was locally injected into the median eminence 20-30μM from the FSCV microelectrode. Further, spontaneous GnRH release was recorded from the median eminence of kisspeptin knockout mice on E18 and P7. To test effect of the inhibitory neuromodulator GnIH on GnRH secretion at 1wk of age, a 1h control recording period in ACSF solution was followed directly by a 1h recording in ACSF containing 1μM GnIH. To test the effect of testosterone, 1wk-old mice were injected SC with 50μg/g testosterone or sesame oil vehicle 4h before brain slice preparation. To test if the GnIH receptor antagonist RF9 could reverse any effect of testosterone, slices from testosterone-treated mice were incubated in ACSF containing 5μM RF9 for 1-3h before and during recordings. RF9 does

not cross the blood brain barrier (239), and this approach circumvented the need for ICV injections in 1wk-old mice.

FSCV Analysis. FSCV data were analyzed using Demon software (Wake Forest University Health Sciences) as described previously (10,163,195). The analysis was preceded by data conversion using IgorPro software. Cyclic voltammograms (CVs) were background-subtracted by averaging 10 background scans. To verify the identity of a spontaneous release peak as GnRH, ten control CVs collected after GnRH was injected into a slice were averaged. Each putative GnRH CV was correlated with this average and was considered to be GnRH if $R^2 \geq 0.8$. This threshold was set to allow for some electrode variability. 89% of CVs passed this test. Changes in GnRH concentration were estimated based on calibration in 5μM GnRH. Two-tailed parametric or non-parametric statistical tests (Graphpad Prism 6) were used where appropriate; $p<0.05$ was considered significant. Data are presented as mean±SEM.

Pituitary Challenge to an Exogenous GnRH pulse. Male mice ages 7, 14, 21d and adults (95.8±2.9d) were randomly assigned to two treatment groups, saline or GnRH. GnRH (Bachem H4005) was diluted to 25ng/ml in 0.9% saline and mice were injected with 150ng/kg or the equivalent volume of saline IP. Mice were decapitated 15min post injection and trunk blood and pituitaries collected. The GnRH dose (150ng/kg) was chosen based on a dose response curve (12.5-200ng/kg GnRH) to result in a physiological LH response in both adults and day 21 pups. Serum LH and FSH were measured in singlicate by the University of Virginia Center for Research and Reproduction Ligand Assay and Analysis Core using the Milliplex Rat Pituitary panel customized for LH/FSH (Millipore). The sensitivity of these two assays, as determined by the Ligand Assay and Analysis Core, is 0.24ng/ml for LH and 2.4ng/ml for FSH. The

intraassay coefficient of variation was 10.3%. All samples were run in the same assay to avoid interassay variability.

Pituitary RNA Extraction and Gene Expression. Pituitaries were stored in RNA Later (Ambion/Life Technologies) at -20C until RNA extraction. RNA was extracted (with on-column DNasing) using RNeasy spin columns (Qiagen). Pituitary RNA (10ng/µl final concentration) and a standard curve of generic adult mouse pituitary RNA (200, 25, 3.13, 0.39, 0.05ng/µl final concentration) were reverse transcribed as described (240). Because of limited RNA yield in neonatal pituitaries, pituitary cDNA (30ng total) and the standard curve (1200, 150, 18.75, 2.34, 0.29ng) were preamplified using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan primer-probes included for preamplification were for mRNAs of: Gnrhr, Lhb, Fshb, Egr1, Fst, Npffr1, Lhb Primary Transcript (PT), Fshb PT and housekeeping mRNAs Gapdh, Ppia, Actin, and Rps29. All Primer-probes were purchased from Integrative DNA Technologies (Table 4-1 and 4-2). Primer-probes were resuspended in Tris-EDTA to 20X (5µM each primer, 10µM probe) as recommended. The components for the preamplification reaction were: 6µl cDNA, 0.2X final concentration of each primer-probe, 12µl 2X preamplification buffer and water to a final volume of 24µl. cDNA was preamplified for 15 cycles using manufacturer recommended cycling conditions. The preamplified cDNAs were then diluted 1:20 with Tris-EDTA, a further dilution of 1:500, for use with the more abundant transcripts (LHb, Fshb, Rps29, Gapdh, Ppia, Actin) was also created. Preamplified cDNAs were stored at -20C until used for qPCR.

Table 1: Integrative DNA Technologies qPCR Assays for Pituitary Gene Expression

Transcript	IDT PrimeTime qPCR Assay	Accession #	Location (bp)	Amplicon size (bp)
Gnrhr	Mm.PT.45.16240237	NM_010323	542-634	93
Lhb	Mm.PT.45.16240237	NM_008497	137-227	91
Egr1	Mm.PT.45.16240237	NM_007913	523-617	115
Fshb	Mm.PT.45.16240237	NM_008045	1-105	104
Fst	Mm.PT.45.16240237	NM_008046	1074-1138	88
Npffr1	Mm.PT.45.16240237	NM_001177511	342-469	128

Table 2: Custom qPCR Primers and Probes for Pituitary Gene Expression

Transcript	Accession #	Location (bp)	Amplicon size (bp)	FWD Primer	REV Primer	Probe
LhbPT	U25145	638-858	221	CGGCAGTACTCGGACCTG	CAGTCTGCATCACCTTCACC	GTCCTAGCATGGTGAGCGGG
FshbPT	NC_000068	107059078-107058934	145	CAAGCCGAAGACTTGAGAGG	GCAAAGCTGGATCAACTTCA	TCAGCTGGTCAGTTTTCACAGTGA
Rps29	NM_009093	119-145	127	TGAAGGCAAGATGGGTCAC	GCACATGTTCAGCCCGTATT	AGTCACCCACGGAAGTTCGG

Quantitative PCR was performed utilizing preamplified PCR product for each of the transcripts as described (240). In short, 5µl of diluted preamplified PCR product were run in duplicate using TaqMan Gene Expression Master Mix (Applied Biosystems) for 40 cycles as indicated by manufacturer. Linearity and parallelism of the preamplification step was confirmed as described (240). Amplicon size was confirmed by agarose gel electrophoresis and sequencing for custom primer-probe sets. Relative gene expression was determined by the delta-delta Ct method (241). Pituitary actin, Gapdh, and Ppia gene expression were regulated developmentally, thus relative gene expression was normalized to Rps29. To avoid intraassay variability all samples were assayed within the same assay.

Differences in normalized relative gene expression were determined by two-way ANOVA with age and GnRH treatment as the main effects, with differences for either GnRH treatment or age were determined *post hoc* by Holm-Sidak test (Graphpad Prism 6).

Pituitary Protein Extraction and Gene Expression. Protein was extracted, quantified and western blots of pituitary protein (20µg) from adult male mice injected with either saline or 150ng/kg GnRH IP, and then euthanized 5, 15, or 30min post injection (n=2-3 per group) as reported (242). As a positive control for GnRH-induced ERK activation (phosphorylation) gonadotrope-derived αT3 cells were treated with either saline or 200nM GnRH for 10min. Blots were immunostained for dually phosphorylated (phospho) ERK1/2 (Cell Signaling cat#4370), total ERK1/2 (Cell Signaling cat#4695), and α-tubulin (Thermo, cat#RB-9281-P1).

Results

Frequency of GnRH release detected by FSCV in the median eminence resembles that of LH release in vivo. We recently adapted the electrochemical method of FSCV to detect GnRH secretion directly in mouse brain slices (10). FSCV directly detects the oxidation of secreted GnRH on carbon fiber microelectrodes. Because the analysis of FSCV requires background subtraction, basal levels cannot be assessed and we thus refer to “changes in GnRH concentration”. We first determined the frequency of spontaneous GnRH secretion detected by FSCV on electrodes placed in the median eminence of brain slices acutely prepared from gonad-intact male mice (Figure 4-1B-D, Figure 4-2D-G). The median eminence is the main site of GnRH release for control of the pituitary gland. In slices from adults, the frequency of spontaneous GnRH release detected by FSCV (as GnRH concentration changes) was 0.3±0.1 events/h. This is similar to reports of LH pulse frequency *in vivo* in adult mice (148), demonstrating a good

match between FSCV measures in the median eminence of brain slices and functioning of the *in vivo* HPG axis.

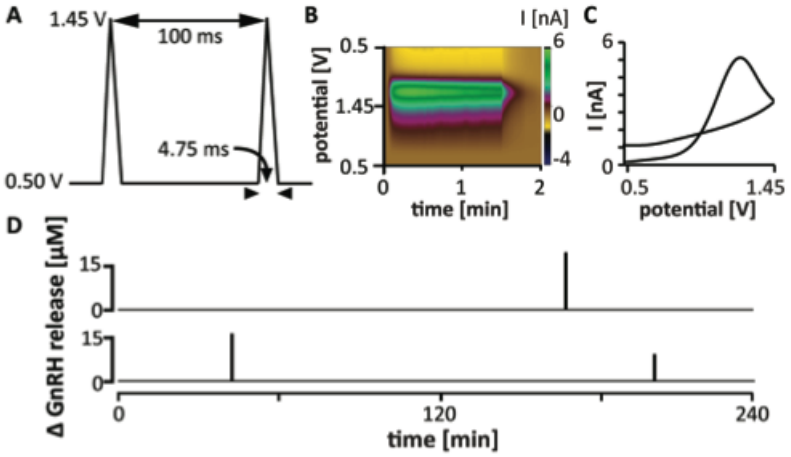

Figure 4-1. Pattern of GnRH release detected in the median eminence of brain slices in adults. A. FSCV waveform. holding potential 0.5V, switching potential 1.45V, scan rate-400V/s (adopted from (12). B. Pseudo-color representation of current changes (color scale) as a function of time (x-axis) and potential (y-axis) for representative spontaneous GnRH release in the median eminence of a brain slice from an adult male mouse. C. Background-subtracted cyclic voltammogram for the spontaneous event shown in B. D. Two representative examples of the pattern of spontaneous GnRH release in adults. Summary data are shown in Figure 4-2 for ease of comparison with other developmental stages.

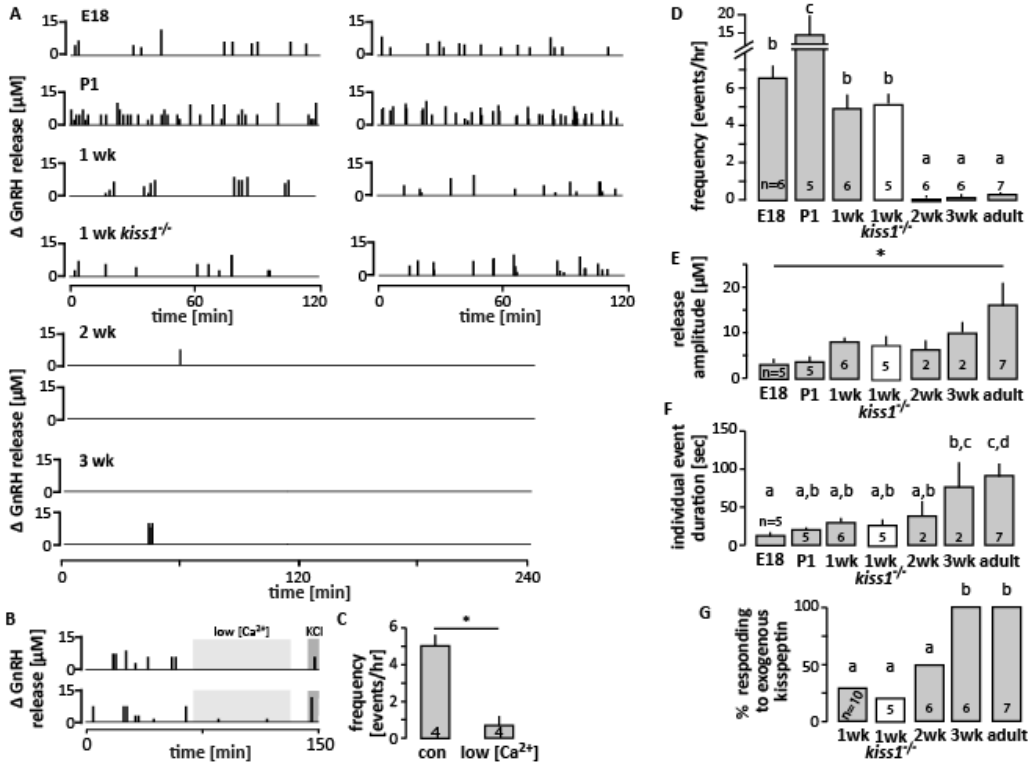

Figure 4-2. Developmental changes in spontaneous GnRH release. A. Two representative examples of the pattern of spontaneous GnRH release at each developmental age studied in wild-type mice and in kisspeptin knockout mice at 1wk. Each vertical bar represents an individual GnRH release event. B. Two representative examples of spontaneous GnRH release in 1wk-old mice in low calcium solution; light gray-low calcium (0.5 mM) buffer present in the recording chamber; dark grey-high KCl (20mM) normal calcium ACSF in the recording chamber to evoke GnRH secretion. C. Mean±SEM frequency of GnRH release in normal and low calcium ACSF, $p<0.05$, paired t-test.D-G. Mean±SEM characteristics of spontaneous GnRH release; numbers within/above bars represent sample size. D. Frequency of GnRH secretion expressed as number of release events per hour; different letters represent statistical significance $p<0.0001$, one-way ANOVA followed by Tukey's multiple comparison test. E. Amplitude of GnRH release; * $p<0.05$, one-way ANOVA followed by Tukey's multiple comparison test. F. Duration of individual events; different letters represent statistical significance $p<0.0001$, one-way ANOVA followed by Tukey's multiple comparison test. G. Responsiveness to 10nM kisspeptin; different letters represent statistical significance $p<0.01$, Chi-Square test.

Changes in spontaneous GnRH release during development. Developmental changes in the pattern of spontaneous GnRH secretion were examined using FSCV starting at embryonic day 18 (E18). Release frequency, peak amplitude and event duration were used to characterize GnRH secretion patterns. A very high frequency release was observed in both embryonic (E18) and neonatal (postnatal day 1, P1) mice (Figure 4-2A, D). At these developmental stages, the frequency of GnRH secretion was higher ($p<0.0001$) than the highest frequency previously reported in adults *in vivo* in any species under any experimental condition (78). Surprisingly, this high frequency GnRH release continued in mice monitored at 7-9d of age, well beyond the very brief perinatal LH rise reported in this species (153). This GnRH release was calcium-dependent, suggesting it is vesicle-mediated and not leakage from damaged tissue (Figure 4-2B,C). In marked contrast to the high release frequency up to 9d of age, at 2 and 3wk the system was essentially shut down; a total of only two spontaneous release events were observed in slices from six mice at each age despite extending recording duration to 4h. All of these preparations released GnRH in response to depolarization with 20mM KCl (Table 4-3). This paucity of spontaneous release was qualitatively different to that in adults, in which spontaneous GnRH release was observed in all slices within 4h. The statistical dilemma of demonstrating differences between numerically low values precluded differentiating release frequency in adults from that at 2 or 3wk of age.

Table 4-3: Spontaneous vs. evoked GnRH secretion

Age group	Number of animals	Animals with spontaneous GnRH release	Animals with KCl-evoked GnRH release
E18	6	6	6
P1	5	5	5
1 wk	6	6	6
1 wk kiss KO	5	5	5
2 wk	6	2	6
3 wk	6	2	6
Adult	7	7	7

With regard to release parameters other than frequency, a steady increase with age was observed in the magnitude of each GnRH release and a corresponding increase in duration of events (Figure 4-2E, F; Figure 4-3), which was shortest at E18 (80% of release events lasting <20s), and increased with age to reach durations >1min in adults (Figure 4-2F; Figure 4-3). Interestingly, age-related changes in spontaneous GnRH release frequency were inversely correlated with responsiveness to kisspeptin, an important excitatory neuromodulator of GnRH release (184,243,244). Kisspeptin injected locally in the median eminence evoked GnRH release in only 2 of 6 of slices from 1wk-old mice; in contrast, 3wk-old and adult slices were 100% responsive (Figure 4-2G).

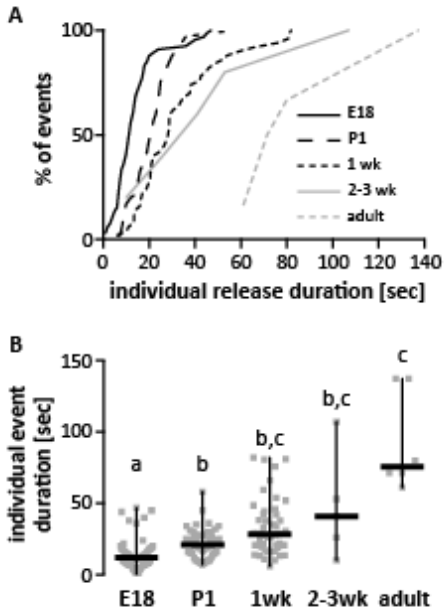

Figure 4-3. GnRH release events appear to consolidate with age, resulting in lower frequency, longer duration release. A. Cumulative distribution function of duration of all individual GnRH events in different age groups. Data for 2 and 3wk-old mice were combined attributable to paucity of events. B. Duration for all individual GnRH events (grey circles), same groups as in (A); horizontal lines- data median; vertical lines- data range; different letters represent statistical significance; $p<0.0001$, Kruskal-Wallis test followed by Dunn's multiple comparison test.

Early prepubertal regulation of GnRH release. To examine why release frequency was so high in early prepubertal animals, we conducted a series of studies in 1wk-old mice; this age was chosen as an example of high frequency release separated from the stress of recent parturition. Low responsiveness to kisspeptin in 1wk-old mice (Figure 4-2G) combined with the high secretory activity of GnRH neurons at this age suggested two possibilities. First, GnRH release may be kisspeptin-independent at this age; second, response to exogenous kisspeptin may be occluded by high endogenous kisspeptin release. We thus recorded spontaneous GnRH secretion from 6-9d old male kisspeptin knockout (KO) mice (189,238). There was no difference in the frequency of GnRH release, event amplitude, duration or responsiveness to kisspeptin between kisspeptin knockout and wild-type animals (Figure 4-2). High frequency GnRH release in 1wk-old male mice is thus kisspeptin independent.

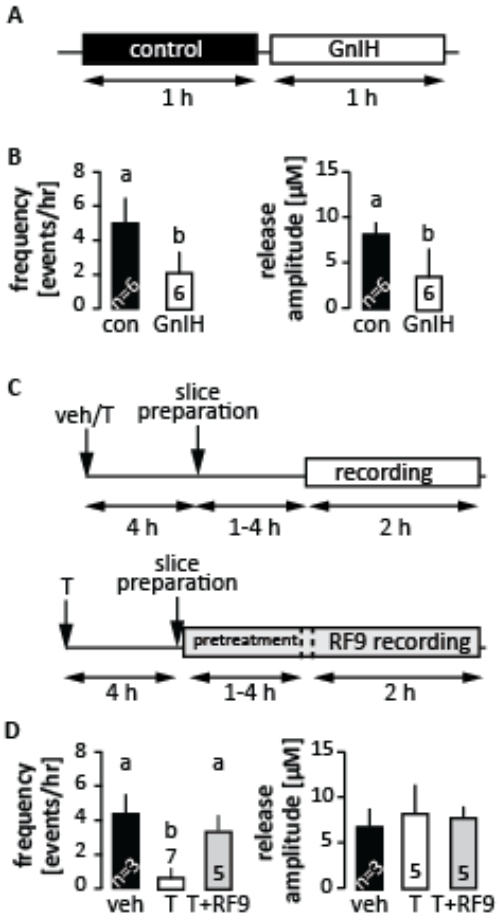

Figure 4-4. GnIH and testosterone inhibition of spontaneous GnRH secretion. A**.** Experimental design for the effect of GnIH on GnRH release in 1wk-old animals; 1h of control recording (white block) followed by 1h of recording in the presence of 1μM GnIH. B. Frequency (left panel) and amplitude (right panel) of spontaneous GnRH release in control recording and after incubation with 1μM GnIH, $p<0.05$, paired t-test. Values are mean±SEM; numbers within/above bars represent sample size, different letters represent statistical significance. C. Experimental design for the effect of peripheral T administration on GnRH release (top panel); black arrows- timing of T/vehicle injection or slice preparation; white bar- timing of recording. Experimental design for the effect of 5μM FR9 on T-induced decrease of GnRH release (bottom panel); black arrows- timing of T administration or slice preparation; grey bar-1-4h of slice pretreatment with RF9 followed by 2h of recording in the presence of RF9B. D Effects of *in vivo* testosterone injection with and without *in vitro* RF9 incubation on frequency (left panel) and amplitude (right panel) of spontaneous GnRH secretion, $p<0.01$, one-way ANOVA followed by Tukey's multiple comparison test.

We next hypothesized that the elevated GnRH secretory activity is attributable to a lack of sufficient inhibition. First, we tested whether gonadotropin-inhibitory hormone (GnIH), a negative neuromodulator of GnRH neurons and the HPG axis (187,218,245), is capable of decreasing GnRH release frequency in 1wk-old mice (Figure 4-4). GnIH (1μM) reduced GnRH release frequency, suggesting a lack of endogenous inhibitory central input at this age (Figure 4-4B, $p<0.01$). We next asked if testosterone inhibited GnRH release at 1wk. Mice received 50mg/g testosterone sc 4h before brain slice preparation (Figure 4-4C). In testosterone-treated mice, GnRH release frequency was reduced ($p<0.01$), whereas release was unaffected in vehicle-injected controls (Figure 4-4D). To examine a possible relationship between these two inhibitory cues in regulating GnRH secretion from the median eminence, we preincubated brain slices from testosterone-injected mice with the GnIH receptor (GPR147/NPFFR1) inhibitor RF9 (5μM) (Figure 4-4C). This treatment restored GnRH frequency to levels observed in untreated mice (Figure 4-4D). These observations strongly suggest testosterone action is at least in part mediated by GPR147 signaling.

Pituitary responsiveness to GnRH. High-frequency GnRH secretion in 1wk-old males was unexpected based on low reported values of LH at that age (246–248). Continuous or very high frequency GnRH infusion leads to desensitization of pituitary response and thus to decreased LH synthesis and release from the pituitary (2,150). Although administration of high frequency GnRH has been used to probe the relationship between frequency and pituitary response, the frequencies used in those studies (up to 5 pulses/h, rhesus monkey)(1) had not previously been observed for spontaneous GnRH release (highest frequencies observed ~3 pulses/h, thyroidectomized sheep)(78), and thus while a useful tool were considered outside the physiologic range. We hypothesized that endogenous production of these high frequencies in 1wk-old mice would induce a

similar pituitary shutdown. We tested pituitary response to an exogenous GnRH pulse over development. Mice received either saline or 150ng/kg GnRH ip; serum and pituitaries were collected 15min later. Serum LH, which reflects responsiveness to GnRH, was below detection levels in both saline- and GnRH-injected males at 1wk, as well as in non-injected newborns (Figure 4-5A). In contrast, mice at all other ages exhibited an increase in LH following GnRH injection ($p<0.01$). There was no clear response to GnRH in serum FSH levels (Figure 4-5B), likely attributable to the more constitutive nature of its release (249).

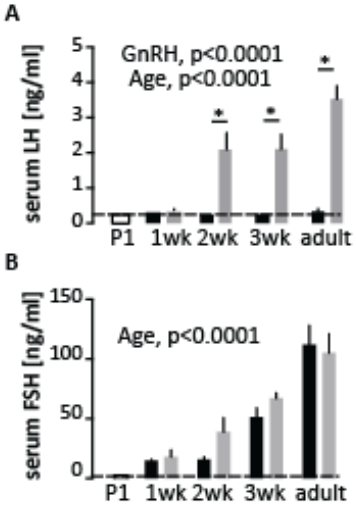

Figure 4-5. Failure of pituitary secretory response to exogenous GnRH at ages exhibiting high spontaneous GnRH release frequency. GnRH-induced LH (A) and FSH (B) release. Data are represented as mean ± SEM, two-way ANOVA followed by Holms-Sidak multiple comparison test (P1 animals were excluded from this analysis as no GnRH challenge was performed in this group). *, $p<0.01$. Note different Y-axis scales in A and B.

To gain insight into possible mechanisms underlying the failure of GnRH to induce LH release, we examined pituitary expression of selected genes (Figure 4-6). GnRH receptor (GnRHR) mRNA is abundant in very young animals, although at lower levels

than in adults, but importantly did not change between 1wk and 2 wk of age despite the acquisition of response to exogenous GnRH (Figure 4-6A). To assess the function of the GnRH receptor, we tested expression of genes targeted by GnRHR signaling pathway. Age-related increases ($p<0.0001$) in expression of LHß and FSHß mRNA were observed, however there were no differences in response to a single GnRH injection (Figure 4-6D, F). Similarly, LHß and FSHß primary transcript expression increases with age ($p<0.0001$), but a single GnRH injection induced LHß response only in adult mice ($p<0.05$) and had no effect on FSHß at any age tested (Figure 4-6C, E). Egr1, an immediate early gene induced by GnRHR signaling, shows similar pattern of expression changes with age ($p<0.0001$), but no response to a single GnRH injection (Figure 4-6B). Interestingly, expression of both follistatin (Fst) and GnIH receptor (Npffr1), which are upregulated by high frequency GnRH pulses (250–253), was increased in early prepubertal groups, and decreased with age ($p<0.0001$) (Figure 4-6G, H); elevation of these transcripts is an independent *in vivo* correlation of high frequency GnRH release. Attempts to examine signaling pathway activation by GnRH were unsuccessful (Figure 4-7), likely due gonadotropes being a small percentage of the pituitary and signaling pathways being more ubiquitous among pituitary cell types than the mRNAs tested, which are enriched in the gonadotrope population. Of note, most studies of GnRH activation of pituitary phospho ERK have been done in gonadectomized/steroid-replaced neuroendocrine suppression models to decrease basal pituitary ERK phosphorylation. The high basal pituitary phopho ERK in the present unsuppressed mice in the present study likely contributed to difficultly in observing ERK activation in response to GnRH.

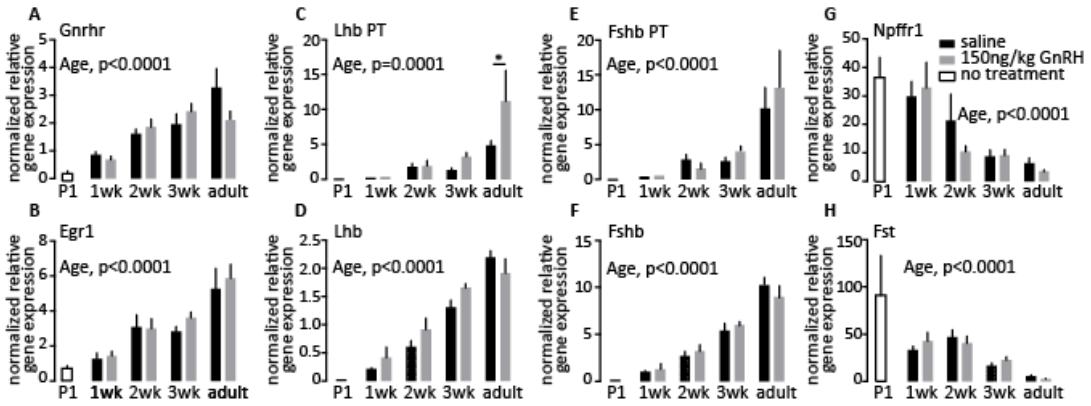

Figure 4-6. Effect of GnRH on expression of selected pituitary genes as a function of age. A-H. Normalized relative gene expressions for GnRH receptor (Gnrhr, A); Egr1 (B); LHß primary transcript (Lhb PT, C); LHß steady state mRNA (Lhb, D); FSHß primary transcript (Fsh PT,E), FSHß steady state mRNA (Fshb, F); GnIH receptor/GPR147 (Npffr1, G); and follistatin (Fst, H). Pituitary gene expression was normalized to ribosomal protein S29 mRNA (Rps29), which did not change with either age or GnRH treatment. Data are represented as mean ± SEM, two-way ANOVA followed by Holms-Sidak multiple comparison test (P1 animals were excluded from this analysis).

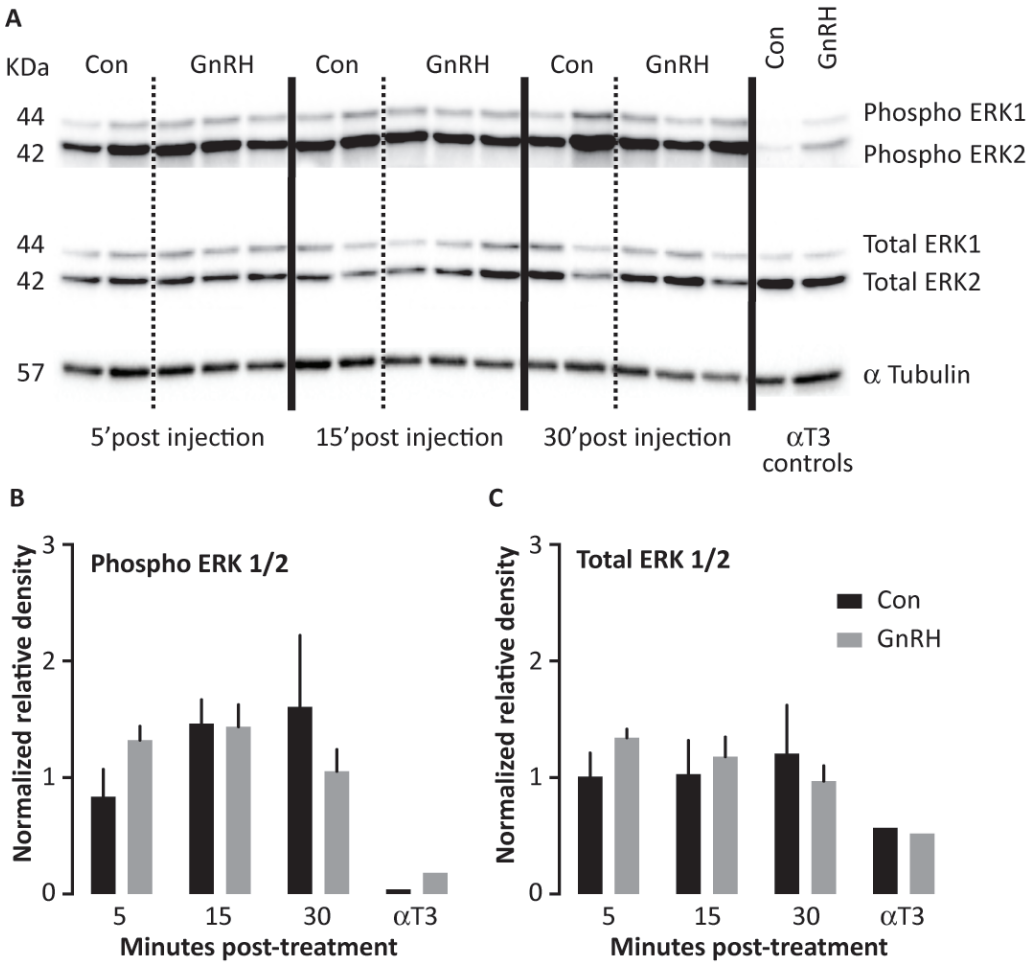

Figure 4-7. GnRH-induced intracellular signaling in mouse pituitaries, *in vivo*. A. Representative western blots of pituitary protein (20μg/lane) from adult male mice injected with either saline or 150ng/kg GnRH, i.p. and then euthanized 5, 15, or 30min post injection (n=2-3 per group) or from αT3 cells treated with either saline or 200nM GnRH for 10min. Blots were immunostained for dually phosphorylated (phospho) ERK1/2, total ERK1/2, and α-tubulin. B-C Changes in phosphorylated (A) and total (B) ERK1/2 were quantified by densitometry.

Discussion

GnRH release before puberty in most species is assumed to be relatively low based on pituitary gonadotropin levels. Here we used FSCV to directly measure GnRH release in brain slices from mice and demonstrate that this neuronal network secretes GnRH at very high frequencies from at least the late embryonic period through the first week of postnatal life.

The present direct observations of GnRH release revealed surprisingly high frequency release in the late embryonic and early postnatal period. The high frequency release persisted for at least a week after the immediate postnatal elevation in pituitary hormones has ceased (153). This frequent secretion may reflect the intrinsic activity of the GnRH system without functional neuromodulatory input. Consistent with this hypothesis, early-life GnRH release is kisspeptin-independent, despite recent evidence that synaptic connections between GnRH and kisspeptin neurons are formed as early as E16.5 (254). Further, endogenous GnIH signaling appears to be absent, demonstrated by the effective suppression of GnRH release by exogenous GnIH. Of interest in this regard, and somewhat counter to a currently prevailing view that GnRH release is kisspeptin-dependent, intrinsic activity is a hallmark of cultured embryonic and immortalized GnRH neurons, which exhibit patterned action potentials, hormone release, and calcium oscillations (64,76,82).

While the GnRH neuronal network present in the brain slices utilized in these studies can generate high frequency release, release at this frequency may not be interpretable by the pituitary as a stimulatory signal as suggested by the lack of LH secretory response to GnRH at 1wk of age. Of note, release pattern from E18-1wk was irregular, in contrast to the typically uniform pattern of release observed in adults in vivo

(5,55,237). This may be an artifact of having removed important regulatory regions during the preparation of brain slices, but may also reflect the immaturity of the afferent network remaining within the slice. Continuous or high frequency GnRH administration has long been known to suppress pituitary gonadotropin release (1,2). The observation that the high endogenous GnRH release frequency at 1wk was associated with pituitary failure to respond to exogenous GnRH with gonadotropin release, suggests the pituitary's requirement for an appropriately patterned GnRH release has a potential role beyond promoting differential gonadotropin release in adults. Specifically, lack of pituitary gonadotropin release when confronted with high frequency GnRH effectively blocks activation of the downstream reproductive system during early postnatal life, preventing inappropriate precocious puberty. The present data appear to contradict a previous study in mice that demonstrated increases in LH in response to GnRH after postnatal d1. We suspect the difference with the present study is in the dose of GnRH, which was 200 times greater in the previous study.

Between 1wk and 2wk postnatal, a marked change in GnRH release was observed. This timing coincides with increased prevalence of GnRH release in response to kisspeptin and with the onset of pituitary release of LH in response to exogenous GnRH. It is possible that during this interval endogenous GnIH signaling has matured and contributes to the marked suppression of endogenous GnRH release, restoring pituitary sensitivity. In this regard, a recent report indicated altered LH profiles during development GnIH receptor knockout mice (220). The high frequency, short duration, irregular GnRH release observed through 1wk of age, became longer and less frequent, organizing more into the pattern observed *in vivo* in adults, arguing for a more mature network remaining in the slice. Upstream neuromodulators including kisspeptin and GnIH may thus play their more important roles in organizing GnRH network activity in

the later stages of the pubertal process, rather than generating GnRH activity *per se*. In addition to the increasing GnRH response to kisspeptin observed here and by others (255), recent studies have pointed to epigenetic changes in the preoptic area and hypothalamus occurring at a similar age (230). Further, ablation of kisspeptin neurons before P20 allows compensation of the overall network so that mice are fertile, whereas adult ablation consistently leads to infertility (256) suggesting a critical role for kisspeptin or other transmitters from these neurons (257) becoming mandatory during the later prepubertal stages.

As with any experimental preparation, it is important to consider the caveats of the approach. FSCV can be fined tuned to detect specific substances; the protocol used in the present study detects no signal in the median eminence of hypogonadal mice even when neurosecretion is evoked by elevated extracellular potassium, demonstrating it is specific for GnRH (10). The signal FSCV detects is attributable to oxidation of GnRH and is thus a direct measure of hormone release. The size of the carbon fiber microelectrode is such that release is likely detected from several terminals. It is important to point out, however, that release events distal to the electrode cannot be detected. This would result in undercounting of release frequency. Because these measurements were made in a brain slice, the pattern of release is from a network that is missing elements that are removed by preparation of sagittal sections. Several observations suggest these measurements are still physiologically relevant. First, the frequency of release detected in slices from adults is similar to published reports of LH pulse frequency (148). Second, release is vesicle-mediated, not just leak from damaged tissue. Third, frequency does not change in a linear manner with age, which one might suspect if an artifact were merely changing over time. Fourth, regulation by testosterone and GnIH suggests at least some relevant afferent systems are preserved. Finally, the lack of LH response to

GnRH at 1wk of age and restoration of that response at 2wk of age are what would be predicted if the frequencies observed *in vitro* were occurring *in vivo*.

Another limitation to the present study is the difficulty in extrapolating these observations between species and even to female mice. Studies of very young laboratory animals, particularly in the first week to 10 days postnatal, are limited. Although there seems to be general agreement that gonadotropin levels are low, there are not any studies of GnRH release during this time for comparison. At slightly older ages, our observations tend to agree with the age-related increase in frequency that has been observed *in vitro* (149) and *in vivo* (237). Comparisons with primates may be of limited value given the relatively unique developmental pattern presented by the prolonged postnatal activation of the hypothalamo-pituitary axis in these species (152). Of interest, the present studies suggest a possible greater similarity between mice and primates at least at the level of GnRH release than was appreciated solely from measures of LH; that is in mice as well as primates, the GnRH system is activated, shut down and then reactivated for completion of the final pubertal transition. Finally, it is important to point out that the developmental stages we examined cannot be broadly compared by age to most other species given the different levels of development at birth. Our sampling window was defined by practical matters; it began at E18 because the GnRH terminals have reached the median eminence in appreciably numbers and because it was possible to make quality brain slices at this age. For some species, the equivalent ages may occur much earlier in gestation.

The possible roles of abundant GnRH secretion long before it can exert downstream reproductive function are of interest to consider. The GnRH pulse generator, which is responsible for driving reproduction in adults, consists not only of GnRH neurons, but incorporates other neuronal and glial elements and integrates a variety of physiological

cues, from energy balance to steroid milieu to circadian information. Such complexity requires proper connectivity among individual elements of the network. Activity-dependent genesis and elimination of synaptic connections is one of the major processes taking place in the developing brain (258). We speculate that the primary role of GnRH secretion from late embryonic through early postnatal development is neuromodulatory and that it is critical for establishment of proper wiring of the reproductive brain. In this regard, recent studies indicate GnRH can be secreted in regions other than the median eminence (10). Both functional (13,259,260) and anatomical studies (261) indicate the presence of GnRHR in the brain. During their migration from the olfactory placode to the diencephalon, prenatally and in adults, GnRH has been proposed to exert neuromodulatory effects on both GnRH and other types of neurons (13,259,260). In this regard, a role of GnRH or GnRH neuronal activity in organizing local circuitry during postnatal sexual development is postulated.

Chapter 5

Conclusions

GnRH neurons generate a pulsatile pattern of GnRH release to the pituitary portal vasculature that is essential for reproduction. Both architecture and function of the GnRH system are remarkably complex and arise from highly orchestrated migratory processes during early development, followed by maturation of the GnRH neuronal network and its connections with neighboring neural and glial circuits that transmit, and most likely enable integration of variety of inputs that regulate GnRH neurons behavior. Alterations to both the development of GnRH system as well as its physiological responses to changing environment can lead to disruption of proper reproductive function. It is crucial then to understand normal physiology of GnRH neurons and mechanisms that govern their particular behavior and function in order to identify potential origins of pathophysiological processes and further to determine targets for therapies or prevention of reproductive diseases.

Detection of GnRH secretion in mammalian models for reproductive research

GnRH neurons have been studied since the XX century, when the role of GnRH in human reproduction and fertility was first discovered (1,2,16). A variety of animal models was utilized in reproductive research: from fish (262), to birds (263,264), to mammals including rodents (265), sheep (265), and other large animals (265), and non-human primates (265). In all of these species a phenomenon of pulsatile GnRH secretion is prerequisite for fertility in both sexes and thus the mechanisms responsible for its acquisition are among major research subjects in the fields of reproductive biology and neuroendocrinology. It is still unclear though, how a relatively small population of roughly 1000 neurons scattered throughout the medial and basal hypothalamus generates coordinated pulses of GnRH release. Studies of regulation of GnRH secretion were especially challenging and limited by existing technical obstacles. Due to the complicated anatomical architecture of the hypothalamo-pituitary-gonadal axis,

particularly the hypophyseal portal vasculature, it is difficult to gain physical access for hormone content measurements. The volume of the blood that contains GnRH is minimal in most mammals used for laboratory research and insufficient for the repeated measurements that are necessary for investigation of patterned release. Studies requiring access to pituitary portal blood are restricted to larger animals such as sheep or monkeys and require complicated neurosurgical procedures making this research time- and resources-consuming. In the case of non-human primates as research subjects, such experiments bring legitimate ethical concerns as our understanding of these animals' neurophysiology and behavior evolves and our ethical standards become higher. Another serious limitation of this approach is the lack of access to transgenic animals. The vast majority of genetic models utilized for study of reproduction, including the most recent tools such as CRISPR-Cas (266), are available almost exclusively in mice. Measurements of GnRH content in portal vasculature provide information about the neuroendocrine output of the GnRH system, but may be unrelated to the release of this peptide in other brain regions. Therefore a method that allows detection of GnRH directly in the brain is necessary to study other sites of GnRH secretion. Microdialysis enables *in vivo* detection of neurotransmitter content in the brain of even small rodents (267,268) with relatively good temporal resolution, but it has several major limitations. Since microdialysis requires perfusion of ACSF through a particular region of the brain it may result in poor spatial resolution. Micordialysis also does not provide information about secretion from individual cells or small groups of neurons within local circuits. Additionally, the neurotransmitter of interest is diluted during the procedure and this may cause limited sensitivity of the method and its inability to detect smaller or spatially restricted but biologically significant release. Microdialysis is also unable to provide information about actual concentration of neurotransmitters, or at least its approximation at or near the site of release. Microdialysis has been used previously to study GnRH

secretion in both rodents (267,269) and monkeys (269) from the median eminence, where GnRH fibers are abundant and packed closely together. This method is not particularly useful to study release of GnRH in other brain regions where GnRH processes and cell bodies, and thus possible sites of secretion, are scattered and fewer in number.

Electrochemical methods have been used previously to detect neurotransmitter release, mainly monoamines such as dopamine or serotonin, with excellent spatial and temporal resolution (270) and enable detection of release from even a single terminal (271). Only a few attempts to use this methodology for peptidergic neurotransmitters detection have been published. These include amperometric GnRH measurements from median eminence of teleost fish (168) and α-melanocyte-stimulating hormone (α-MSH) from rat melanocytes (167). Unfortunately amperometry may not be a reliable method for GnRH detection. Amperometry records the oxidative current at only one particular voltage. It is usually set at the potential at which substance of interest generates the highest oxidative current when compared to other voltages. However, it is still possible that other substances, which have peaks of oxidative current at a different potential can still be oxidized and therefore contribute to false-positive signal. In the median eminence, as well as other hypothalamic locations, multiple peptides can be secreted and much better specificity for detection tool is required.

In Chapter 2 of this dissertation I develop a method that utilizes fast-scan cyclic voltammetry, or FSCV, to detect GnRH release in mouse brain sections. I provide evidence that FSCV is a reliable, specific method for direct GnRH release measurements in the median eminence. Further, as evidence for excellent spatial resolution, I demonstrate for the first time that the preoptic area secretion can be detected near the appositions of GnRH soma-process or two GnRH processes. Since

FSCV sampling rate is much more frequent that in any other method currently available, secretion events as short as few seconds, and potentially shorter, can be easily detected. This gives FSCV significant advantages over existing tools for GnRH release measurements, allowing for very specific, localized readouts of both spontaneous as well as evoked GnRH secretion. To obtain informative cyclic voltammograms FSCV data processing requires subtraction of background signal. Therefore any basal GnRH release, if it occurs, will not be captured. Only changes in concentration can be measured and it is important to keep in mind that numerical values of detected GnRH release are relative to unknown basal level. This is one of the major limitations of this methodology and may be particularly important for the interpretation of spontaneous GnRH release pattern. However, the GnRH system still appears to be a good subject for electrochemical studies due to its episodic nature of biological activity. Further, studies of evoked release in response to precisely-timed stimuli do not seem to be affected by this relative nature of FSCV measurements. Another potential limitation is the loss of carbon fiber microelectrode sensitivity over the first ten minutes of constant voltage cycling. This loss elevates the threshold for GnRH detection and consequently decreases FSCV's ability to detect smaller amplitude GnRH release events. This is most likely due to the interaction of GnRH oxidation products with the surface of electrode (272). Decline in sensitivity can lead to an underestimation of both frequency as well as amplitude of recorded GnRH secretory events. Chemical modifications to the surface of carbon fiber microelectrodes have been previously utilized to improve their parameters for detection of specific substances (273) and a similar approach could be a solution to decreasing sensitivity of currently used microelectrodes for GnRH detection.

With proper choice of recording parameters FSCV could be, at least theoretically, employed to detect any electrochemically-active substance, for example short peptides

that contain tryptophan and/or tyrosine in their amino acid sequence. As mentioned previously, both median eminence and hypothalamus, brain regions that take part in setting and maintaining body homeostasis, are highly populated by neurons that release many different neuropeptides (132,274–276), and some of them might be candidates for FSCV detection. Since it is difficult to predict solely from peptide sequence if such detection is possible and what parameters are appropriate for a stable signal, individual peptides have to be studied on a case-by-case basis. Both tryptophan and tyrosine can have multiple products of oxidation (277), which can interact with the electrode surface. In summary electrode desensitization is a likely problem in attempts to utilize FSCV for peptide detection. It has been shown previously that α-MSH desensitizes carbon fiber microelectrodes during amperometric recordings at 850mV within seconds (278), suggesting that the same problem may occur for voltammetry. Stringent preliminary studies of exogenous peptides *in vitro*, followed by validation in biological tissue preparations (e.g. brain slices, *in vivo*) are necessary for any novel adoptions of FSCV for biological research.

Detection of GnRH secretion by FSCV described in Chapters 2-4 is performed in brain slice preparation. The utility of this method is not restricted to *ex-vivo* applications, and *in vivo* recordings are also possible (279) and could be employed in future studies of the GnRH pulse generator.

GnRH secretion and action potential firing: Revealing regional brain differences

While performing biological validation of FSCV as a tool for monitoring GnRH secretion I discovered, as described in detail in Chapter 2, that GnRH release is not restricted to the median eminence sites but also occurs spontaneously in the preoptic area. I used a specific mouse model (37) recapitulating changes in feedback mode exerted by estradiol

during normal estrus cycle of female mice. In this particular paradigm, a shift from negative to positive estradiol feedback to GnRH neurons that normally occurs between diestrus and proestrus phase is elicited daily in ovariectomized mice that received estradiol replacement. This model is characterized by daily changes in both LH levels as well as electrophysiological properties of GnRH neurons (37). In the mornings a negative estradiol feedback leads to low serum LH levels as well as low firing frequency of GnRH neurons as recorded at their cell bodies (37). In the afternoons a positive estradiol feedback takes action and induces both higher LH and high frequency of GnRH neuron firing (37). The frequency of GnRH release in the median eminence recorded in this model revealed analogous changes, which is consistent with the common understanding of GnRH frequency coding of LH synthesis and release from the pituitary gland. What is particularly perplexing, preoptic area GnRH secretion did not follow this pattern and there were no changes in release frequency observed between morning and afternoon. Secretion remained at relatively low levels that were comparable to those observed during negative feedback in the median eminence. In other words, GnRH secretion in this region did not correlate with either changes in firing rates observed previously and recorded mainly in the preoptic area GnRH neurons (37), or with secretion detected under the same conditions in the median eminence. This suggests that mechanisms governing GnRH release in the preoptic area may be different from those in the median eminence and that GnRH can play a role other than classical neuroendocrine function if released to the brain. Moreover, regarding the lack of parallel changes in firing and release in the preotic area during different estradiol feedback stages, the dependence of GnRH secretion in this region upon spike generation becomes ambiguous. Chapter 3 brings novel insights into our understanding of how GnRH secretion in those two brain regions could be controlled independently of each other. Data presented there suggest that while secretion of GnRH from the median

eminence is mostly action potential firing-dependent, release in the preotic area can easily occur without spiking activity in the local circuitry. GnRH secreted from the median eminence to the hypophyseal blood is likely released in a coordinated manner necessary to generate sufficient changes in GnRH levels that could be interpreted by pituitary gonadotrophs. In this regard, action potential-dependent release should be considered as a major mechanism underlying GnRH secretion from this location. GnRH release in the median eminence must produce an intermittent pattern, although not all GnRH terminals have to contribute to each episode of release, or even to the entire episode of release. Since FSCV detects GnRH events occurring in proximity to the recording electrode, secretory events observed in the median eminence most likely originate from multiple terminals juxtaposed to the recording site, rather than from the entire region. Using several carbon fiber microelectrodes positioned in spatially non-overlapping local clusters of GnRH terminals, the contribution of individual release sites to the generation of GnRH pulses can be studied. One of the interesting questions that such experiment could help to answer is how much coordination between multiple release sites in the median eminence exists, especially between the two hemispheres.

Studies described in Chapter 3 reveal a different contribution of action potential firing to GnRH secretion between the preoptic area and the median eminence. However, they do not precisely define if the firing of GnRH neurons is prerequisite for release in any of those regions, particularly median eminence, or how the pattern of spiking activity of GnRH neurons shapes their secretory output. This could be possibly studied by performing dual electrophysiological and electrochemical recordings to monitor spontaneous firing and release from GnRH neurons. Design and interpretation of such experiments requires consideration of existing methodological constraints. In the preoptic area GnRH release can be detected only at the close appositions of two GnRH

neurons, but either or both of them could be a source of secretion. Simultaneous monitoring of firing and release requires thus an active suppression of vesicle release from one of those cells in order to enable proper data analysis and interpretation. Such blockade could be achieved by increasing calcium buffering in one neuron. Dialysis of calcium chelators through micropipette in whole-cell mode can potentially prevent activation of vesicle membrane fusion machinery and thus neurosecretion (280). Additionally, injection of hyperpolarizing current may be useful to prevent firing activity as well as calcium influx through voltage-gated calcium channels. Both of those manipulations will be technically challenging. Their effectiveness may be hampered due to peripheral localization of release site relative to the cell body, where such interventions are feasible. Although it has been studied in other types of neurons (280), the time necessary for chelating agents to dialyze entire GnRH neuron, including terminal of interest, is unknown. Diffusion of chelating agents needs to be tested first by including a dye in the recording pieptte, along with the effectiveness of such treatment in preventing secretion from GnRH neurons. Both extracellular recording of firing activity that will be performed on the second neuron, as well as monitoring of release, can last for hours in a healthy slice, which suggests that even if prolonged treatment with intracellular chelators is necessary, the experiment is feasible.

In the median eminence similar studies will meet two major types of difficulties. One is strictly technical and refers to electrophysiological recordings in this brain region. Anatomy of the GnRH system dictates the necessity to record from GnRH neuron terminals because the region lacks cell bodies of those cells. Characteristics of the tissue, which is remarkably dense and adheres easily to the electrode, prevent the utilization of classical extracellular recordings, due to difficulties in the establishment of a stable seal of the electrode on the GnRH neuron terminal. Stable seal is prerequisite for

high quality recording and proved to be virtually impossible on the median eminence terminals of GnRH neurons. Despite multiple attempts to overcome this obstacle that were taken in the Moenter laboratory, no prolonged monitoring of GnRH neurons firing at their terminals in the median eminence was achieved. The utility of the sharp electrode technique, method that was not tested in the median eminence, is one of the few remaining alternatives. Once the method for stable recording of firing properties of GnRH neurons terminals in the median eminence is attained, both spikes and GnRH release could be monitored in parallel. The second major difficulty relevant to this experiment is data interpretation. As mentioned previously, GnRH release detected by FSCV in the median eminence most likely originates from multiple terminals adjacent to the recording electrode and there is no information regarding exactly which terminal or terminals contribute to the given release event. Unless firing of GnRH neurons contributing to the secretory event is coordinated, information about spiking activity of only one GnRH fiber may be irrelevant to the pattern of observed release if the release does not originate, or only partially originates from the terminal chosen for electrophysiological monitoring. No studies of firing properties of multiple GnRH neurons in the median eminence are available and we lack the information about possible coordination of firing in this region. Data available for GnRH neurons cell bodies located outside of the median eminence suggests that no direct electrical/synaptic connectivity between neighboring GnRH neurons exists (72). This may be irrelevant for the median eminence and studies of the local GnRH circuitry in this region are in a high demand. An alternative approach to recordings of individual neurons in brain slice preparation is an *in vivo* approach with utilization of multiple unit activity (MUA) electrophysiology in combination with FSCV. Targeting MUA recordings exclusively to GnRH terminals may not be possible, since other types of neurons could be active in the same anatomical location. Two-photon imaging might be a better and

more specific option for monitoring GnRH neuron electrical activity due to the availability of mice expressing eGFP under the GnRH promoter (6) to allow for easy visual identification of GnRH neurons, including their terminals. It has to be kept in mind that *in vivo* recordings would require prolonged anesthesia, which could potentially interfere with results and make their interpretation inconclusive. Different classes of anesthetics have been shown to impact ovarian physiology (281) as well as GnRH neuron behavior (282) and therefore are typically not used in electrophysiological studies of the reproductive system.

The role of GnRH neuron firing in regulation of GnRH secretion could be also studied in a genetic model, in which firing of action potentials by GnRH neurons is impaired. Although such model has not been developed yet, a possible candidate can be identified. Action potentials generation depends primarily on voltage-gated sodium channels, or NaVs, (283), and different subtypes may be responsible for generation and shaping of action potentials in different types of neurons. A preliminary study of gene expression profile in GnRH neurons reveals that one of the major sodium channel subunits expressed by GnRH neurons is NaV1.6 (data unpublished). This channel type has been previously demonstrated to be a primary channel underlying action potentials initiation and propagation in cerebellar Purkinje neurons (284). Their action potential kinetics are similar to those exhibited by GnRH neurons (72), and may suggest that this channel type could indeed be critical for proper spike generation in GnRH neurons. Specific knockout of NaV1.6 (or *scn8a*) in mouse GnRH neurons does not have any obvious deleterious effects on fertility. Such mice breed normally and females exhibit normal estrus cyclicity, as demonstrated on Figure 5-1.

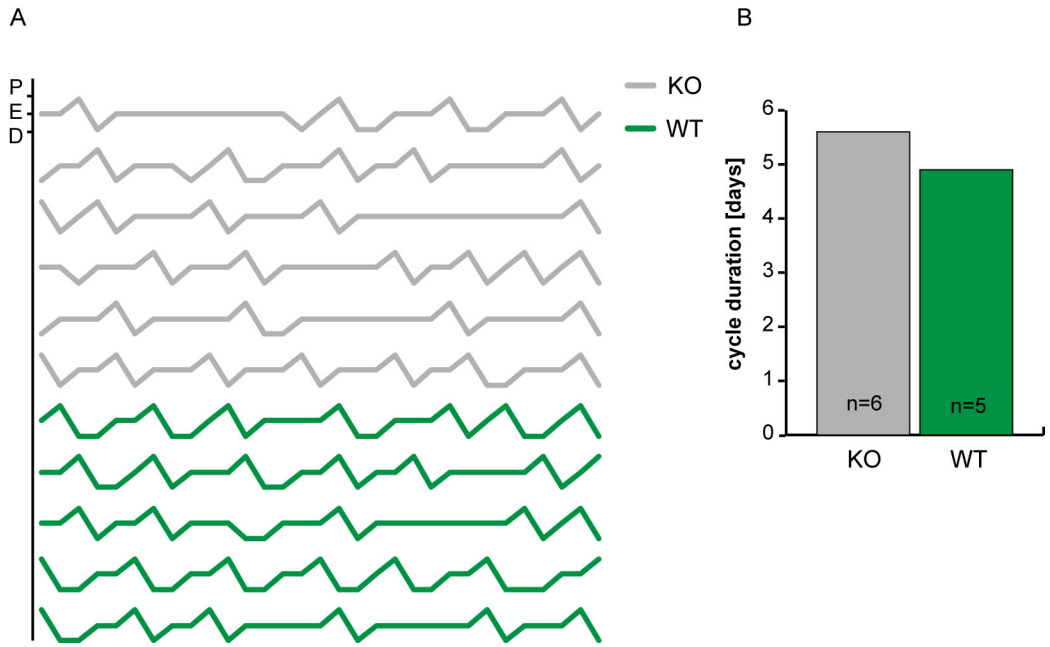

Figure 5-1 Estrous cycles of mice lacking NaV1.6 in GnRH neurons do not differ from wild type mice. A. One month duration of estrous cycle monitoring in GnRH-specific NaV1.6 knockout (KO, grey) and wilt type (WT, green) mice; each line represents cycles of one animal; P= proestrus, E= estrus, D= diestrus; B. Mean duration of an estrus cycle in knockout (KO, grey bar) and wild type (WT, green bar) mice; numbers in bars indicate sample size.

This may suggest that action potential firing in GnRH neurons is not crucial for reproductive function, or that this particular sodium channel is not primarily responsible for proper spiking activity of GnRH neurons. Further studies are necessary to determine the role of NaV1.6 in GnRH neuron electrophysiology. Recordings of firing properties will provide information on whether initiation and propagation of action potentials in GnRH neurons in GnRH-specific NaV1.6 knockout animals are impaired in comparison to wild type controls. Further, if any differences are revealed, their implications on the pattern of spontaneous GnRH secretion should be studied by utilizing FSCV in both the median eminence as well as the preoptic area.

Regional differences in GnRH secretion between the preoptic area and median eminence revealed in Chapter 3 strongly suggest that local neuronal and/or glial circuitry

may play important role in modulation of GnRH release and that their molecular and functional organization may differ between the two regions. One possible class of components engaged in region-specific regulation of GnRH release are cannabinoids. Studies of their effects on the reproductive system have shown their negative influence in many species including rodents and primates, as well as humans (285–290). Previous research suggests that those effects are primarily confined to the brain (291) and may be both directly and indirectly affecting GnRH neurons (292). Interestingly, intracerebroventricular administration of Δ^9-tetrahydrocannabinol (THC) in rat decreased serum LH levels, suggesting depression of GnRH secretion from the median eminence, but GnRH content measured in the medial basal hypothalamus was increased (291). This may implicate differential region-specific effects that cannabinoids may exert on GnRH secretion. My pre-doctoral studies revealed that cannabinoids can modulate GABA-ergic synaptic inputs to GnRH neurons in the preoptic area in an activity-dependent manner and by engaging local neuronal and glial circuitry (121). Although their effect on GnRH neuron electrophysiological behavior was very subtle, it does not preclude more significant consequences on GnRH secretion. Previous data from the preoptic area indicate that cannabinoids can be synthesized by both immortalized (292) and native GnRH neurons (121,293) and influence the physiology of a local network. Neither the role of GnRH release in this circuitry nor the effects of endogenous cannabinoid synthesis and their role in regulation of GnRH secretion have been tested. *In situ* hybridization studies indicate that cannabinoid receptor 1 (CB1) is expressed in brain regions overlapping with GnRH neurons locations, specifically in the preoptic area (292,294), but very little colocalization with GnRH was observed in mice. This suggests that GnRH neurons in the preoptic area that are also capable of producing cannabinoids might auto-regulate their function, which is hypothetically GnRH release activity, by retrograde CB1 receptor signaling to their local afferents. The CB1 receptor is also

abundant in the median eminence; interestingly it is found mostly in the terminal parts of axons present in this region (295), potentially in GnRH terminals among others, supporting the evidence that cannabinoids can directly and/or indirectly modulate GnRH secretion for neuroendocrine function. Detailed studies of how cannabinoids could regulate secretory activity of GnRH neurons in the preoptic area along with the median eminence are of interest, especially in the context of increasing recreational cannabinoid consumption by both adult and adolescent humans (296).

Another interesting aspect of the regulation of GnRH neuron physiology is the role of sex steroids. Broad electrophysiological research has been conducted in different animal models to test the effects of steroid hormones, both gonadal and centrally produced, on the electrophysiological properties and behavior of GnRH neurons (27,28,37,41,88,297). Gonadal steroids provide significant feedback to GnRH neurons, regulating both their firing activity (27,28) as well as fast synaptic transmission that these cells receive through the afferent network (29,30,37,38). Additionally, they influence glial architecture in the GnRH neurons local circuitry (36,110). It is essential to note here that the great proportion of these studies, with exception of glial sheathing of GnRH terminals, was performed in somatic region of GnRH neurons, mostly in the preoptic area, and it may be hard to drive any conclusions about anticipated behavior of distal terminals in the median eminence. Although the effects of gonadal sex hormones on GnRH secretion to the pituitary portal vasculature are well described (52,55), their role in regulation of GnRH release for neuromodulatory function as well as possible modulation of GnRH release by centrally-produced steroids remain elusive. As discussed in the previous section, preoptic area GnRH release in female mice does not appear to be affected by the feedback mode estradiol exerts on median eminence secretion. It clearly indicates that different mechanisms might govern release in this brain region. Moreover, it may

suggest that GnRH secreted in the preoptic area is either estradiol-insensitive or simply does not change with its feedback status. Ovariectomized female mice with only estradiol replacement were used in those experiments and thus lack of other gonadal hormones could affect the outcome. As a follow-up study, spontaneous preoptic area GnRH secretion should be investigated in naturally cycling animals. This could reveal the effects of changing hormonal background under more physiological conditions. The role of specific hormones or their combinations can be further elucidated in ovariectomized animals along with different steroid milieu replacement. Studies of possible sex differences are desirable as well. Since only females and not males exhibit complex cyclical changes in the activity of hypothalamic-pituitary-gonadal axis. It is reasonable to hypothesize that the physiology of local GnRH circuitry implicated in regulation of GnRH neurons activity, both electrical and secretory, may differ between the sexes. Preoptic area GnRH secretion in males may follow notably different pattern than in females and be less sensitive to steroid milieu since in males gonadal steroid levels exhibit much less fluctuation and any changes are not as precisely timed as in females.

Steroid hormones are also produced centrally (298) and play a variety of functions directly in the brain (299,300), even in regions that are primarily not related to reproduction but may exhibit sex-specific functional features (301). Studies by Sun et al. (41) indicate that acute estradiol treatment that mimics action of locally synthesized hormone activates L- and R-type calcium currents conducted by voltage-gated calcium channels in GnRH neurons. Since voltage-gated calcium channels can regulate neurosecretion (302), centrally produced estradiol can potentially affect GnRH release. Acute estradiol-evoked calcium currents were present in mice that were both ovariectomized and untreated or treated with estradiol, which suggests that systemic

estradiol did not influence this effect. Of note, all recordings were performed in the preoptic area and similar effects in the median eminence are only hypothetical. Therefore, FSCV can be utilized to study possible implications of acute estradiol treatment on GnRH secretion in the preoptic area and the median eminence in both sexes to evaluate possible sex differences in modulatory role of neurosteroids on central component of the reproductive axis.

Development of the adult pattern of GnRH secretion and the role of neuropeptides

Findings of the studies presented in Chapter 4 demonstrate surprising changes in the pattern of GnRH secretion from the median eminence during sexual development of male mice. A very high frequency of GnRH events observed amid the perinatal period and persisting until the age of one week appears to be a result of insufficient inhibitory signaling at the early stages of life. This coincides with relative unresponsiveness of GnRH secretory activity to kisspeptin, a major stimulator of adult reproductive neuroendocrine axis. Together these data suggest that during early development GnRH neurons exhibit very high intrinsic secretory activity, most likely necessary for proper wiring of the reproductive brain, and inhibitory signals emerge later to prevent hyper-stimulation of GnRH circuitry when it becomes responsive to the kisspeptin signaling. It can be also speculated that GnRH released in the median eminence may have a neuromodulatory role before its endocrine function arises because it does not seem to exert downstream effects of the pituitary. It still remains unknown what triggers the switch from active to quiescent secretory status occurring between the first and second week of postnatal life in these animals. It is possible that intrinsic activity of GnIH in the brain increases during that time and could be tested by utilization of RF9, a GnIHR inhibitor. Interestingly, the high frequency GnRH release from the median eminence during the peri- and neonatal period is accompanied by lack of pituitary response, and

LH secretion driven by GnRH is activated simultaneously with hypothalamic silencing. This brings into question whether emergence of pituitary responsiveness to GnRH signaling is just a release from high frequency GnRH-triggered desensitization, or whether some other process independent of hypothalamic activity mode guides this change. The mechanisms determining the lack of the gonadotrophs' ability to respond to GnRH before two weeks of age are still unclear. Although gene expression data suggest this system might be desensitized by high frequency GnRH and additionally inhibited by GnIH, the exact mechanisms are elusive. It could be tested in gonadotroph cell cultures derived from mice at different ages, if perfusion with variable frequencies of GnRH triggers any response at both secretory and molecular level. Studies of cell signaling *in vivo* are challenging, especially in very young animals such as those utilized in discussed research. *In vitro* approaches may enable better-controlled, more specific tests that could bring more precise answers than current data does.

Findings of GnIH ability to inhibit high frequency GnRH secretion in early prepubertal animals support and further extend recent studies demonstrating that male GnIHR knockout mice exhibit elevated levels of LH before puberty, but their sexual maturation does not seem to be affected by this defect (220). Interestingly, GnIHR knockout animals eat more and thus gain weight faster than their control littermates (220), and consistent with previous findings that GnIH is implicated in feeding behavior (303). This could mask the effects of GnIH on puberty timing, since increased body mass can advance this process (304,305). GnIH has been shown to regulate the reproductive axis on both central (187,188,218) and peripheral levels (223,306,307). Dissection of its function in different components of the HPG axis by knocking down GnIHR specifically in brain, pituitary, or gonads and by controlling the timing of genetic manipulation by inducible knockouts will further advance our understanding of GnIH's role in

development of reproductive function.

Studies presented in Chapter 4 clearly indicate that neuropeptides other than GnRH that are involved in control of reproduction in adult and most likely pubertal animals, do not play an active role in regulation of early life activity of GnRH system. It appears that their signaling is either absent although GnRH neurons can respond to it (e.g. GnIH), or they do not respond in a manner characteristic for later developmental stages (e.g. lack of response to kisspeptin). This does not preclude an active role of other neuropeptides that have not been investigated in this research. Other possibilities include neurokinin B and dynorphin that are primarily implicated in positive and negative control of reproduction (308). Both of these peptides are coexpressed with kisspeptin in arcuate nucleus (200) and loss of function mutations of both neurokinin B and its receptor in humans have been shown to be responsible for pubertal failure (309). Neurokinin B is also implicated in puberty in rodents (310). Very little is known about function of these peptides before puberty onset. Pharmacological tools such as agonists and antagonists of neurokinin B and dynorphin receptors as well as animal genetic models can be utilized to investigate how these peptides could affect the pattern of GnRH secretion.

My current studies of development of GnRH secretion were confined to males, but since mammalian reproductive physiology is by its nature sexually dimorphic, similar experiments in female mice will need to be conducted. Preliminary data from prenatal females at E18 suggest that, at least during this early stage in development, the pattern of GnRH release in the median eminence is similar between the sexes (Figure 5-2A). Interestingly, kisspeptin knockout animals exhibit a pattern of GnRH pulsatility in the median eminence similar to that in wild type controls, and again no sex differences are apparent at this stage of research (Figure 5-2B and C). Further investigation in other age groups is necessary to validate the hypothesis that in early prepubertal mice the

secretory activity of GnRH system does not differ between males and females and that similar mechanisms govern its development. It is possible that changes in pattern of GnRH secretion that differentiate sexes arise later in life, most likely around puberty onset. The timing of first signs of puberty as well as acquisition of full reproductive maturation differ between sexes (311), and it is thus likely that diverse mechanisms and/or pace of physiological changes drive pubertal process in male and female mice.

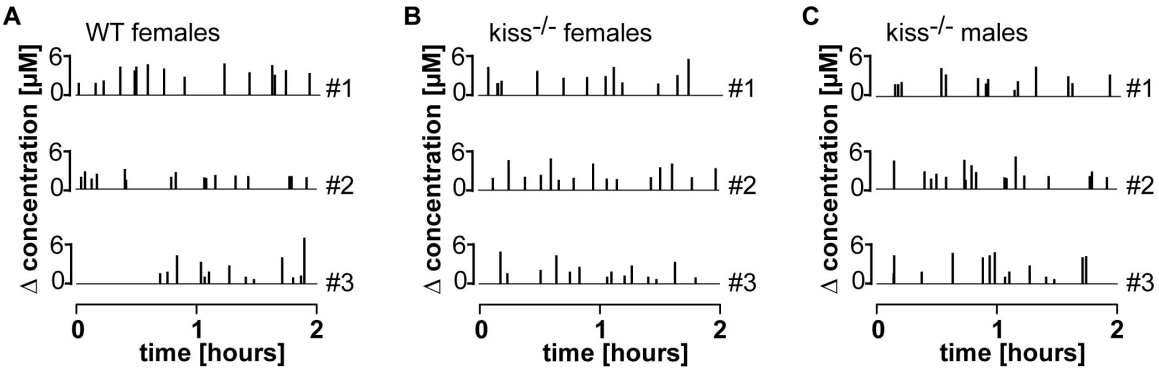

Figure 5-2. Pattern of a spontaneous GnRH secretion in prenatal mice is not different between the sexes and appears to be kisspeptin-independent. A-C. Examples (#1-3) of spontaneous GnRH release in wild type female mice (A) and kisspeptin knockout females (B) and males (C). Each vertical bar represents an individual GnRH release event.

As discussed earlier, the median eminence is not the sole site of GnRH release. Neuromodulatory GnRH release in the preoptic area may be another component of maturation of the reproductive brain. It is completely unknown when such release may be initiated and detailed studies at different stages of both embryonic and postnatal development are required. Interestingly, only a very small fraction of the entire GnRH neurons population by brain graft is capable of restoring fertility in adult hypogonadic mice entirely lacking GnRH peptide (312), which suggests that an even more scattered network than the typical ~800 to 1000 (25) neurons is sufficient for fertility. This indicates significant redundancy in the reproductive system and may suggest that GnRH signaling during development may not be necessary for fertility. Similar redundancies are

observed in other components of network that regulates GnRH neuronal function, specifically in kisspeptin signaling (256), but it still exists in normal brain. Although preoptic area GnRH signaling may be redundant for fertility, it still may be required for normal, not compensatory development of the reproductive brain. Since adult preoptic area secretion appears to be regulated independently of median eminence release, it is reasonable to hypothesize that a similar phenomenon will be observed in young animals. GnRH secretion in this region may be relevant to shaping local circuit connectivity and function, similar to how we think this happens in the median eminence, but the pattern of this release and whether it follows similar changes as the median eminence is difficult to predict.

Other interesting features of GnRH neuronal networks which either have not been studied or on which very little information is available in young animals is electrical activity of GnRH neurons as well as synaptic connectivity (261). Preoptic area and median eminence recordings of spontaneous GnRH secretion in the surge model, described in detail in Chapter 2, suggest that spiking activity of GnRH neurons in the preoptic area corresponds better to release in the median eminence than local release. The question arises, whether a similar phenomenon is true for perinatal or juvenile animals. Studies of both prolonged action potential firing and fast synaptic transmission will help to answer those questions. Increased electrical activity and excitability in the developing brain has been well-described (313,314) in other types of neurons and shown to participate in proper network formation (258). It is very likely that it is true for GnRH neurons as well. Additional studies of anatomical connectivity of GnRH neurons with their afferent network at different stages of development may strengthen their relevance and further expand the electrophysiological studies of neurotransmission. Currently available genetic tools enable investigation of monosynaptic retrograde

connections to the target population of neurons by using modified rabies virus systems. One such system, developed in the Moenter laboratory, utilizes adenoassociated virus and modified rabies virus specifically in GnRH cells and was shown to be effective in studies of monosynaptic connectivity in adult GnRH neurons (315). The same technology could be applied to investigate GnRH neurons connectivity in prepubertal and pubertal animals. Design of such studies will require careful timeline planning for virus injections, since they have to be separated by approximately one week, a time that is sufficient for significant functional changes in the behavior of developing GnRH pulse generator and potentially for reorganization of neural circuitries.

Once the normal developmental changes of GnRH release and acquisition of mature pattern of secretion are characterized, potential critical periods for proper development of this system can be identified. Maturation of the reproductive axis is regulated by variety of factors including energy balance and metabolic cues (275), sleep (316), hormonal balance (317) and others. Many reproductive disorders are known to have or are suspected of having developmental origins and defining critical periods for action of potential disruptors can help develop prevention as well as early intervention strategies for treatment. Polycystic ovary syndrome, or PCOS, is one of the most common causes of infertility in women (318). It is characterized by elevated androgens and insensitivity to progesterone feedback resulting in disrupted cyclicity and ovulation and is often accompanied by metabolic syndrome (318). Human data suggest that the disease originates before sexual maturation (319). The Moenter laboratory developed a mouse model of prenatal androgenization (PNA) recapitulating many of the phenotypes demonstrated by PCOS women (320,321). It can be used to study the differences between normal and PNA mice during development of the reproductive axis, specifically the changes in pattern of GnRH secretion and pituitary response and whether any

defects may be corrected before the animals reach puberty. These studies suggest viable interventions for humans, before full manifestations of disease occur.

Final conclusions

Studies presented in this dissertation provide two major contributions to the field of neuroendocrinology. First, they bring a new method for direct GnRH measurements in brain tissue that enables very detailed characterization of the pattern of GnRH release thanks to its excellent spatial and temporal resolution. None of the studies described in Chapters 3 and 4 of both spontaneous and evoked GnRH secretion would be possible in mice without this method, since existing techniques do not have comparable parameters for either specificity or sensitivity. In the future it may help address one of the most important questions in the field about the relationship between firing and secretory activity of GnRH neurons.

The second set of important contributions of this work are the novel insights it brings to our understanding of development of GnRH system as well as mechanisms governing episodic GnRH release. These provide surprising findings of unexpectedly high secretory activity long before GnRH neurons exert their reproductive function and further suggest that balance between excitatory and inhibitory neuromodulators is important for proper sexual maturation. These insights also extend our current knowledge of regional specialization of GnRH neurons and provide evidence supporting the hypothesis that, when released directly to the brain, GnRH plays a role other than a direct control of downstream components of HPG axis. This work also clearly suggests that the relationship between firing activity of GnRH neurons and/or their local circuitry and secretory output of GnRH system may be physiologically labile and further studies are required to understand the perplexing nature of this relationship.

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