ABSTRACT

Extensive studies over the last decade have identified the molecular circadian clockwork controlling intrinsic daily rhythms. Nonetheless, the molecular cellular mechanism underlying ultradian rhythm remains largely unknown. Therefore, I intended to focus on gonadotropin-releasing hormone (GnRH) pulse generator driving episodic GnRH secretion with approximately one-to-two-hour interval, a classic example of ultradian rhythm. In spite of its discovery in the early 1970s, the mechanism for the GnRH pulse generator still remains elusive. In Chapter 1, I aimed to decipher the ultradian rhythm of GnRH pulse generator in the view of dynamicity in GnRH gene expression. For this purpose, I cultured preoptic area (POA) GnRH neurons derived from neonatal transgenic mouse harboring a GnRH promoter-driven destabilized luciferase construct (GnRHp-dsLuc). Furthermore, the regulation of GnRH pulsatility by kisspeptin, a strong activator of GnRH neuron, was explored. In Chapter 2, I investigate whether a defective circadian clock influences the GnRH pulse generator in order to elucidate a possible link between the circadian oscillator and ultradian rhythm. For this purpose, pulsatility of GnRH neurons derived from knockout mouse lacking *Bmal1*, a key transcription factor of core clock machinery, was studied.

1. In Chapter 1, I investigated the temporal dynamics of GnRH gene transcription with its secretion. For this purpose, I obtained hypothalamic POA slices derived from neonatal GnRHp-dsLuc transgenic mouse. Real-time bioluminescence imaging revealed that GnRH promoter activities show a pattern of ultradian oscillation with 10-hr period under the basal condition, which is relatively longer than the period of GnRH pulse generator *in vivo*. GnRH neuronal population stochastically synchronized at an approximately 2-hr interval and was only partially associated with pulsatile GnRH secretion. Intermittent application of kisspeptin, a strong

activator of GnRH neuron, markedly induced transient increases in promoter activity with robust reinforcement of the pulsatile secretion of GnRH which responded to each kisspeptin pulses. Pharmacological manipulation revealed that newly synthesized proteins may be required to maintain pulsatile secretion of GnRH and that retrograde signaling may exert an effect on GnRH gene transcription. Taken together, these results suggest that pulsatile kisspeptin tone strongly influence the GnRH pulse generator, leading to the temporal pattern of GnRH transcription and secretion.

2. In Chapter 2, I investigated the hierarchical interaction between circadian and ultradian rhythms. In order to monitor GnRH pulsatility in mouse lacking functional circadian clockwork, POA slices were obtained from GnRHp-dsLuc TG mice crossbred with a mouse lacking the *Bmal1* gene were used. GnRH neurons in *Bmal1*-deficient knockout (KO) mouse exhibited comparable level of basal promoter activity and hormone contents of GnRH. While spontaneous bouts of GnRH promoter activity in *Bmal1*-deficient GnRH neurons were essentially the same as those in wildtype neurons, kisspeptin-induced pulsatile GnRH secretion was significantly attenuated. Response to continuous application of kisspeptin in *Bmal1*-deficient cultures suggests that attenuation of pulsatility may not result from the lack of basal *Gpr54* expression or its downstream signaling pathways.

In conclusion, the cellular circadian clockwork is needed to generate the hypothalamic GnRH pulse generation probably through the processing, storage, or secretion of the GnRH peptide together with GnRH transcription.

Key words: gonadotropin-releasing hormone (GnRH), pulse generator, ultradian rhythm, kisspeptin, gene expression, episodic secretion, circadian rhythm, hierarchical interaction, Bmal1

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BACKGROUND AND PURPOSE

BACKGROUND

1. Biorhythm

1.1. Rhythmic processes in the biological system

The harmonious function of organisms depends on the proper coordination of biological processes in regard to both spatial and temporal organizations. Biological rhythm is a special form of temporal organization in which the set of elementary biological processes are regularly repeated with a similar pattern, and at a given interval (Goldbeter et al., 2008). The biological rhythms serve a variety of essential functions for the biological processes: adapting to the external environmental changes, temporal encoding of the information in neural and humoral signals, and recognizing the passage of time.

From the periodic generation of action potentials in neurons or cardiac cells to the hormone secretion and circadian rhythms, many key cellular processes exhibit biological rhythms (Fig. 1). Biological rhythm also occurs as emergent property, for example the estrous cycle and annual rhythms.
Multiplicity of biological rhythm can be simultaneously observed in biological processes. For instance, high-resolution profiling of glucocorticoid (GC) levels in the systemic circulation clearly exhibits that the circadian rhythm tracks an underlying ultradian activity (Lightman and Conway-Campbell, 2010). The apparent peak and trough of circadian activity

actually reflect changes in the amplitude of the pulses, with the largest pulses coinciding with the awakening responses and the onset of circadian activity. Similar overlapped rhythms are also reported in many endocrine hormone secretions (Veldhuis et al., 2010). In a budding yeast, several ultradian rhythms with different periods, 16 hrs, one hour and four minute rhythms are simultaneously observed in metabolic oscillation, which originate from partial cell-synchrony, cell-cycle as sociated rhythm, and cellular or mitochondrial oscillation, respectively (Lloyd et al., 2008).

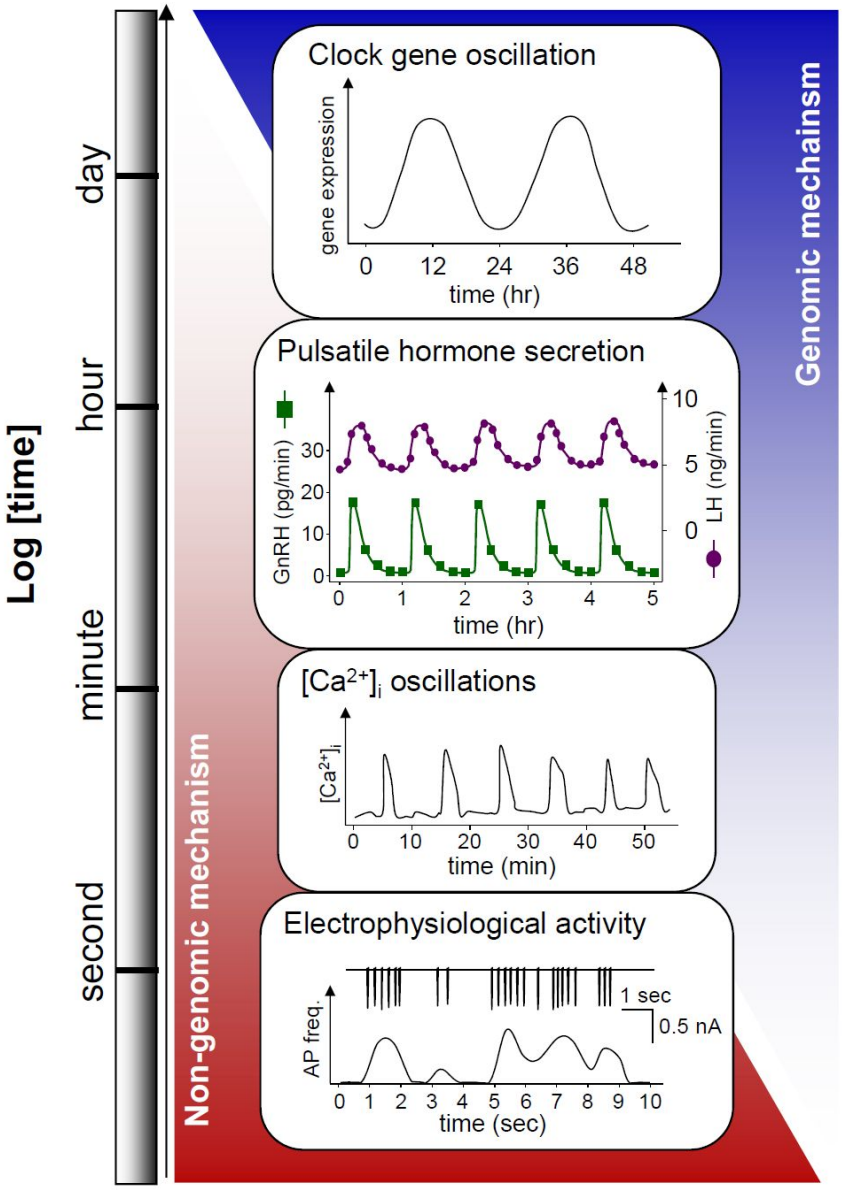
1.2. Various types of biological rhythms

The biological rhythms range over many orders of period from sub-seconds to years. In general, biological rhythms can be classified into ultradian (multiple occurrence in a single day), circadian (with period of approximately a day), and infradian rhythms (extending over more than a day) (Lloyd et al., 2008).

Circadian rhythm is a biological rhythm with a period of approximately a day. It is maintained by intrinsic genetic machinery, with adjustment with external environment. The molecular bases of circadian rhythm have been particularly well studied compared with any other biological rhythms (Goldbeter, 2008). In mammals, the circadian timing system has a hierarchical organization, composed of a master clock in the suprachiasmatic nucleus (SCN) and peripheral, or local clocks in almost every other organ (Dibner et al., 2010). The master clock in the SCN is

synchronized to 24-hr light/dark cycle, and then entrains the phase of cell-autonomous molecular oscillators in peripheral tissues through a variety of neuronal and humoral cues (Son et al., 2011).

Figure 1. Diverse biological rhythms. Various types of rhythms are manifested in the biological system. Representative biological rhythms in GnRH neurons and HPG axis are presented in log scale. Expression of clock genes is circadian. Hormone secretion, especially GnRH and LH, pulsates in every one to two hours. Intracellular calcium levels oscillate with approximately several minutes period. Occurrence of action potential in a single GnRH neuron exhibits a pattern of burst with second-range intervals. Molecular cellular mechanisms underlying the diverse biological rhythms are only partly understood. Genomic mechanism is usually associated with longer-period rhythms, while non-genomic mechanisms are associated with shorter-period rhythms. GnRH: gonadotropin-releasing hormone, LH: luteinizing hormone.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Non-genomic mechanism
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Pulsatile hormone secretion
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LH (ng/min)
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[Ca2+]i
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time (min)
Electrophysiological activity
1 sec
0.5 nA
AP freq.
0 1 2 3 4 5 6 7 8 9 10
time (sec)

A variety of biological rhythms including neural rhythms, calcium oscillations, biochemical oscillations, and gene expression, display a pattern of ultradian rhythm, which oscillates with periods shorter than a day (Goldbeter, 2008; Lloyd et al., 2008). These ultradian rhythms are diverse in their period which ranges from sub-seconds to hours in their function. They are evident from the simpler to higher form: cAMP oscillation associated with spore formation in fungi, metabolic cycle observed in yeast, fluctuation in the hormone level, and electroencephalogram (EEG) during sleep.

Over four decades ago, ultradian fluctuations in reduced pyridine nucleotide concentration was observed in yeast cells, thus providing one of the earliest patterns of ultradian oscillation in the biological system (Laxman and Tu, 2010). Subsequently, several groups have studied the ultradian oscillation of yeast metabolism, which is primarily determined by oxygen utilization, under high cellular density and limiting glucose levels. The period of oscillations extended from approximately 40 min to more than 10 h, depending on the strain and culture conditions (Laxman and Tu, 2010). Interestingly, microarray analyses revealed that the majority of yeast genes exhibited cyclic changes in mRNA expression levels along with cyclic oxygen consumption (Klevecz et al., 2004; Tu et al., 2005). In addition, level of cellular metabolites, such as amino acids and their precursors, nucleotides and TCA cycle metabolites, also showed a robust oscillation in phase with metabolic cycle (Tu et al., 2005), suggesting that overall cellular activities are temporally coordinated according to the metabolic cycle.

It is worth noting that ultradian rhythms are commonly observed in the neuroendocrine system. After discovery of pulsatility in the circulating luteinizing hormone (LH) levels (Dierschke et al., 1970), secretion of most endocrine hormones, such as follicle-stimulating hormone (FSH), prolactin, thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), rennin, insulin, glucagon, parathyroid hormone (PTH), cortisol, sex steroids, and aldosterone, has been shown to oscillate with a pattern of ultradian rhythm, albeit with different period and regularity (Veldhuis, 2010). Distinct ultradian rhythms observed in the neuroendocrine system are thought to serve an important role in the action of hormone.

Biological rhythms having periods longer than the circadian range are called infradian rhythms. Estrous cycle and seasonal rhythm are the most evident and well-known examples of the infradian rhythm. Seasonal rhythm, the so-called circannual rhythm, is clearly demonstrated in the migration of seasonal birds and reproduction of seasonal breeders (Goldbeter, 2008). Estrous cycle refers to the periodic changes in the reproductive system characterized by changes in the secretory profiles of estrogen, progesterone and gonadotropins, which oscillate in 4~5 days in rodents and approximately a month in primates and humans (Knobil, 1980; Christian and Moenter, 2010). Interestingly, infradian rhythms are often discovered to be intertwined with circadian or ultradian rhythm. Ovulation, one of the indispensible phase of estrous cycle, occurs only in a given time window of a day, thus is closely associated with circadian rhythm. Additionally, these

periodic changes of hormonal profiles are dictated by pulsatile GnRH, a prominent example of ultradian rhythm.

1.3. Molecular oscillators driving biological rhythm

In mammals, the circadian rhythm is driven by multiple oscillators that are organized in hierarchy – central and peripheral clocks (Dibner et al., 2010). The central pacemaker resides in the SCN of the hypothalamus. The master clock in the SCN is tuned to external environmental signals, majorly by receiving light input by innervation from the retinohypothalamic tract (Stratmann and Schibler, 2006). After a finding that even immortalized fibroblasts harbor functional molecular clockwork (Balsalobe et al., 1998), the molecular circadian clock has been believed to reside in most mammalian tissues and organs.

The molecular circadian clock, which resides in almost every cell, is primarily based on two interlocked transcription-translation feedback loops (Fig. 2). A core loop involves circadian locomotor output cycles kaput (*Clock*), brain and muscle ARNT-like protein 1 (*Bmal1*), period homolog 1 (*Per1*), *Per2*, and *Per3*, Cryptochrome 1 (*Cry1*), and *Cry2* (Takahashi et al., 2008; Bellet and Sassone-Corsi, 2010). CLOCK and BMAL1 form a heterodimer and bind to E-box elements to activate transcription of clock-controlled genes (CCGs) that include *Per*s and *Cry*s. PER and CRY proteins form a repressor complex which subsequently translocates into the

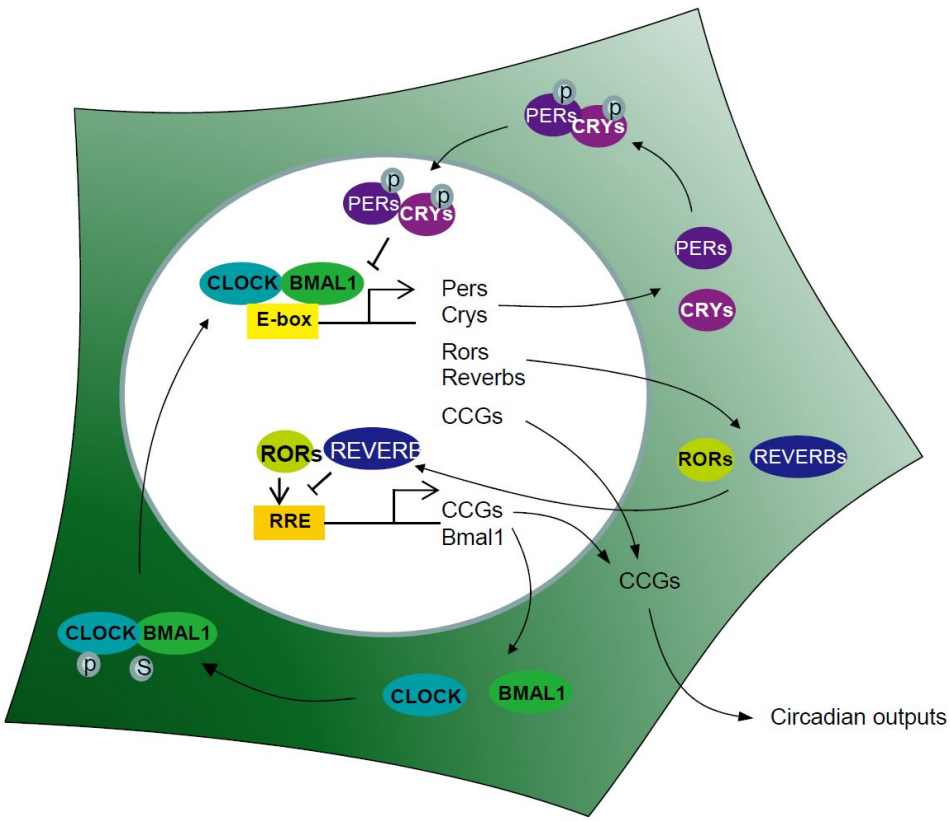
nucleus and inhibits CLOCK-BMAL1-mediated transcription of CCGs including PERs and CRYs themselves, thus forming a feedback loop.

An auxiliary feedback loop is composed of nuclear receptors reverse erythroblastosis virus-a (*Rev-erbα*), *Rev-erbβ* and retinoic acid receptor-related orphan receptor-α (*Rorα*) (Asher and Schibler, 2011; Takahashi et al., 2008). The activator RORα competes with the repressors REV-ERBα and REV-ERBβ for the occupancy of RORE elements within promoter and enhancer regions of *Bmal1*. The auxiliary loop is completed through expression of the *Rev-erbα* and β of which transcription is directly activated by CLOCK-BMAL1.

Additionally, several regulatory processes are superimposed on the transcription-translation feedback loop for fine-tuning of the circadian clock machinery. It has been shown that post-transcriptional regulation, including processing of mRNA and regulation through miRNA (Cibois et al., 2010), post-translational modification, such as phosphorylation, acetylation, sumoylation, ubiquitination, and protein degradation (Bellet and Sassone-Corsi, 2010; Gallego and Virshup, 2007) contribute to proper oscillation of the molecular circadian machinery. The most prominent example of post-translational modification can be found in the post-translational regulation of BMAL1 protein. BMAL1 is a target for various kinases, including casein kinase Iε (*CKIε*) and glycogen synthase kinase 3β (*GSK3β*), and exhibits a pattern of circadian phosphorylation in a variety of tissues and fibroblasts (Bellet and Sassone-Corsi, 2010). BMAL1 is also a substrate for sumoylation (Cardone et al., 2005; Lee et al., 2008), ubiquitination (Kwon et

al., 2006; Lee et al., 2008) and acetylation (Hirayama et al., 2007), thereby modulating its various cellular processes, including transcriptional regulation, cellular localization and protein half-life.

Figure 2. Molecular circadian clock. Cellular molecular circadian clock constitutes of interlocked transcriptional/translational feedback loop. Heterodimeric transcription factor CLOCK and BMAL1 transactivates E-box mediated transcription. The promoters of Periods and Cryptochromes, negative regulators of core clock machinery, contain E-box elements and thus are activated by CLOCK:BMAL1 complex. PERs and CRYs form complex and translocate into the nucleus to inhibit E-box mediated transcription. On the other hand, the expression of RORs and REV-ERBs are activated by E-box-dependent transcription. RORs increase the expression of BMAL1 via RRE in the promoter of Bmal1, while REV-ERBs suppress the expression of BMAL1. These two interlocking feedback loops generate robust circadian oscillations of circadian clock genes. Many genes which harbor E-box or RRE in their promoter region are regulated by circadian clock genes, therefore contributes to circadian outputs.


p
PERs
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Circadian outputs

Recently, circadian rhythm is proposed to be tightly linked with basic cellular activities, such as epigenetic regulation. Epigenetic regulation is composed of a number of mechanisms, including methylation of DNA, microRNA-mediated pathways, and post-translational modifications of histone. The most striking example is the rhythmic recruitment of CLOCK-BMAL1 to the Dbp promoter (Ripperger and Schibler, 2006), of which activation is associated with trimethylation of lysine residue at position 4 of histone 3 (H3K4 trimethylation), a marker of open chromatin, and H3K9 acetylation while H3K4 dimethylation is associated with the repressive phase. In this regard, it is interesting that CLOCK has intrinsic histone acetyl transferase activity (HAT) with a preference for acetylating lysine residues in H3 and H4 (Doi et al., 2006). Additionally, the HAT activity of CLOCK appears to be balanced by the nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase sirtuin1 (SIRT1), which is an important regulator in epigenetic regulation and cellular metabolism (Asher et al., 2008; Nakahata et al., 2008).

A growing body of evidence suggests that transcriptional oscillation is involved in the molecular basis of ultradian rhythms. GnRH promoter activity was reported to exhibit hourly ultradian fluctuation by using real-time monitoring of GT1-1 cells (Nuñez et al., 1998). Similar fluctuation in transcriptional activities was also reported in prolactin and growth hormone promoter activity (Takasuka et al., 1998; Norris et al., 2003). The ultradian rhythms including transcriptional oscillations have also been observed in somitogenesis (Dequeant et al., 2006), NF-kB (Hoffmann et al., 2002), and

p53 (Geva-Zatorsky et al., 2006), which are important for the embryo development, immune response, and cell growth/death, respectively (Mengel et al., 2010).

Similar to the circadian rhythm, self-sustained intrinsic timing mechanism operates for the seasonal rhythm (Hazlerigg and Loudon, 2008). Direct evidence for the existence of self-sustained circannual oscillators has been obtained by keeping animals in constant environmental conditions, including light/dark period and temperature, for more than years, and observing continued cycles in such seasonal parameters as migratory restlessness, hibernation and seasonal moulting (Gwinner, 2003; Kondo et al., 2006). Circannual oscillation often appears to be irregular from cycle to cycle and act as damped transients, suggesting that circannual oscillators are relatively weak compared with circadian oscillators. In addition, seasonal rhythms can be entrained by day-length change (photoperiod). Consistent with circadian rhythms, seasonal rhythms can be entrained to a brief exposure to the external cue (Hazlerigg and Loudon, 2008). Analysis of circannual pattern of blood prolactin level in sheep revealed that phase-response of circannual rhythms is independent of the circannual phase at which the resetting stimuli is imposed, which is characteristic of a weak oscillatory system (Lincoln et al., 2006).

2. GnRH pulse generator: A representative example of ultradian rhythm

2.1. GnRH neurons in control of the pituitary LH function

GnRH neurons produce and secrete GnRH decapeptide, pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. They form the final common pathway in the central regulation of reproduction by integrating the signal from external and internal environment and orchestrating the activity of hypothalamus-pituitary-gonadal (HPG) axis.

A half-century ago it was hypothesized by Geoffrey Harris that neurohumoral factors from the hypothalamus govern pituitary hormones in the hierarchy of the reproductive physiological cascade. In 1971, the primary amino acid sequence of GnRH responsible for evoking LH release was independently identified by Roger Guillemin and Andrew Schally, and subsequently it was confirmed that GnRH is released into the portal vessel with concordant changes in plasma LH levels. Thereafter, GnRH decapeptide has been identified in a number of animal species from protochordates to mammals.

GnRH gene was first isolated from a human genomic DNA library by using synthetic nucleotide oligomers against the coding sequence of GnRH decapeptide (Seeburg and Adelman, 1984). The GnRH gene is composed of 4 short exons and 3 intervening introns spanning approximately 4.5 kb (Fig. 3). Coding sequence resides in exon 2, while GnRH-associated

peptide (GAP) is encoded through exon 2-4. At a posttranslational level, the removal of intron A is a key regulatory event of efficient GnRH expression. While introns B and C contain consensus splice sites, intron A harbors a suboptimal 3' splice site that does not allow efficient splicing (Seong et al., 1999). Translation of the mature GnRH mRNA results in the production of preprohormone consisting of 92 amino acids. After series of posttranslational processing, including cleavage by several peptidases, N- and C-terminal modification, the mature form of GnRH decapeptide is formed.

The GnRH neuronal population is localized in the hypothalamus, which is distributed in a scattered manner without forming a discrete nucleus. They extend caudally from the diagonal band of Broca, past the optic chiasm, and into the medial basal hypothalamus. In rodents, GnRH cell bodies are most abundant in the preoptic area (POA) and the organum vasculosum of the lamina terminalis (OVLT) (Kim et al., 1997; Moenter, 2010). The targets of axonal projection of GnRH neurons are relatively widespread and reside in distant regions. Importantly, GnRH axons project and terminate at the external zone of the median eminence, which is responsible for the neuroendocrine regulation of the pituitary gonadotropes (Kim et al., 1997; Herbison, 2006).

The unique distribution of GnRH neurons is attributable to their developmental origin. GnRH neurons are originated from the olfactory placode and subsequently migrate into the hypothalamus (Wray, 2010). After this migration, GnRH cell bodies are localized in the forebrain region,

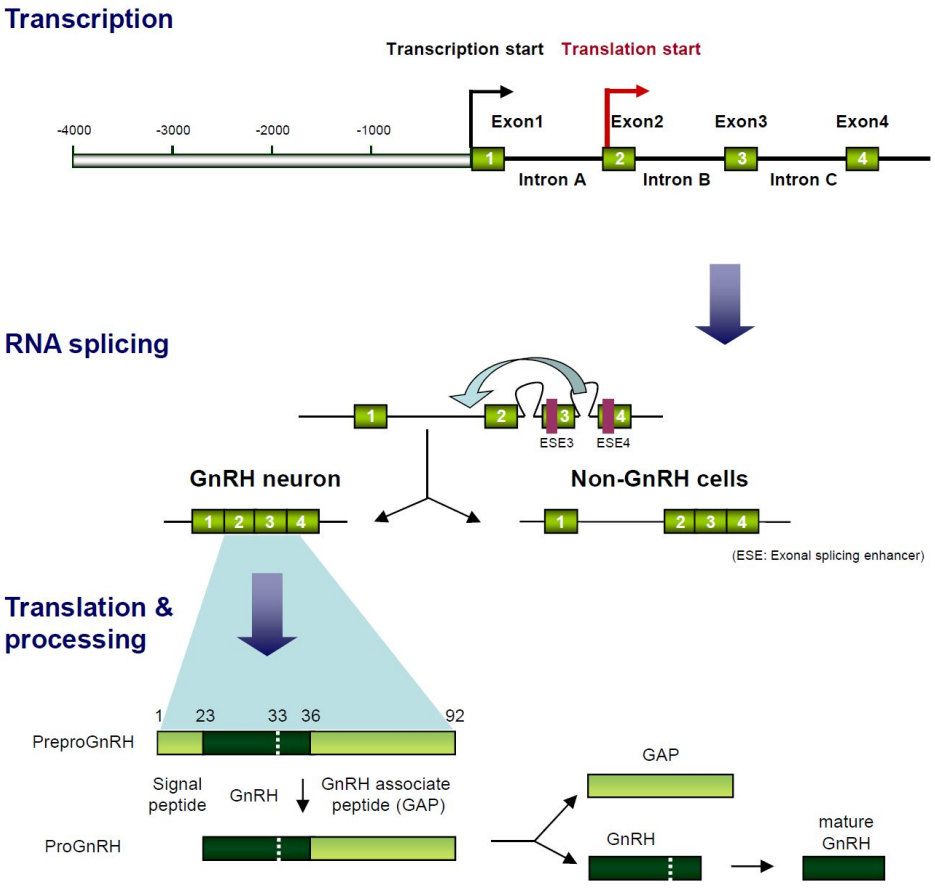
especially preoptic area and anterior hypothalamus, in a scattered manner along their migratory pathway.

2.2. Pulsatile GnRH secretion

GnRH is secreted into the portal vessels in a pulsatile manner. Knobil and his colleagues first reported the pulsatile pattern in LH levels in the circulation of ovariectomized rhesus monkeys (Dierschke et al., 1970). GnRH levels in pituitary stalk blood exhibited a consonant fluctuation with the pulsatile gonadotropin secretion (Carmel et al., 1976), thus suggesting a critical role of pulsatile GnRH release in the process. Its physiological implication was also elegantly demonstrated by Knobil and his colleagues in the recovery experiment using hypothalamus-lesioned rhesus monkey (Belchetz et al., 1978). Since then, pulsatile GnRH/LH secretion has been found in a number of mammalian species such as rats, guinea pigs, pigs, ewes, cows, and horses (Maeda et al., 2010).

In spite of striking rhythmicity and physiological implication, the molecular and cellular source of GnRH pulse generator still remains unknown. Earlier ablation and transplantation studies suggest that molecular machinery for GnRH pulse generator may reside in the medial basal hypothalamus (MBH), and more specifically, the arcuate nucleus (ARC) (Knobil, 1980; Maeda et al., 2010). Additionally, electrical recording of multiunit activity (MUA) suggested that electrophysiological correlates of the ultradian, pulsatile pattern of GnRH release lie within the MBH and that

Figure 3. Structure of GnRH gene and its expression. Mammalian GnRH gene is composed of 4 exons and 3 intervening introns, which contains coding sequence starting from exon 2. The first intron is excised in a GnRH neuron-specific manner, and crucial in translation of GnRH mRNA. Translation of GnRH mRNA results in preproGnRH, which is conjugated to N-terminal signal peptide and C-terminal GnRH associate peptide (GAP). After enzymatic cleavage of both signal peptide and GAP, mature decapeptide form of GnRH is ready to be secreted.


Transcription
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-3000
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-1000
Exon1
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Exon3
Exon4
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Intron A
Intron B
Intron C
RNA splicing
ESE3
ESE4
GnRH neuron
Non-GnRH cells
(ESE: Exonal splicing enhancer)
Translation &
processing
1
23
33
36
92
PreproGnRH
Signal
peptide
GnRH
GnRH associate
peptide (GAP)
ProGnRH
GAP
GnRH
mature
GnRH

the pulsatile GnRH release and MUA may be driven by a neuronal oscillator with an approximate period of 1 hour.

On the other hand, more recent studies based on immortalized GT1 cell lines and primary culture of embryonic olfactory placode as well as GnRH-GFP transgenic mouse, suggested that GnRH neuron itself harbors a mechanism for driving GnRH pulse generator (Herbison, 2006; Maeda et al., 2010). GT1 cells, which were established from the hypothalamus of transgenic mouse expressing T-antigen in a GnRH neuron-specific manner (Mellon et al., 1990), show pulsatile GnRH release *in vitro* with a regular pulsatility (Martinez de la Escalera et al., 1992). Explant culture of embryonic olfactory placode, which is enriched with GnRH neurons, exhibited pulsatile GnRH secretion after 2-3 weeks of cultivation (Terasawa et al., 1999a).

2.3. Hypothetical mechanisms of the GnRH pulse generator

Extensive studies so far have focused on the underlying mechanism, which still remains largely unknown. GnRH neurons exhibit spontaneous oscillation in a variety of cellular activity, including electrical activity, calcium oscillation, and oscillatory promoter activity. The hypothetical mechanisms of the GnRH pulse generator are majorly based on either non-genomic or genomic events.

Non-genomic mechanism is focused on the electrical or calcium oscillations of GnRH neurons. Studies using both whole-cell and

extracellular recordings found that a majority of GnRH neurons in acutely prepared brain slice as well as those mechanically isolated from brain slice or in cultures of embryonic mouse and primate neurons, fired spontaneous action potentials or spikes (Moenter et al., 2003). More importantly, the majority of examined GnRH neurons exhibited bursts of action potential, which are composed of several action potentials and intraburst resting period. Intraburst resting period usually extends over seconds range. Organization of action potential burst and intraburst resting period has been suggested to be responsible for the generation of GnRH pulses (Nunemaker et al., 2003).

GnRH neurons exhibit calcium oscillation with intervals of approximately 8 min and these become synchronized every 50 min in monkey placode cultures (Terasawa et al., 1999b). Similar calcium oscillation in GnRH neurons and their synchronization were also observed in mouse olfactory placode cultures, albeit shorter interval (Moore et al., 2002). Oscillation of intracellular calcium levels appears to be the consequence of episodic electrical activity, at least in GT1 cells (Constantin and Charles, 1999). Overall, it is suggested that episodic firing and calcium oscillation of GnRH neurons are closely related to pulsatile secretion of GnRH decapeptide (Herbison, 2006; Nuñez et al., 2000), although their relevance *in vivo* is yet unclear.

On the other hand, it is hypothesized that oscillation in the biosynthesis of GnRH would contribute to the GnRH pulse generator. Among multiple biochemical steps comprising biosynthesis cascade of GnRH decapeptide,

transcription of GnRH gene was observed to be oscillatory in GT1 cells (Nuñez et al., 1998). By using continuous real-time monitoring of single GT1 cell, it was revealed that promoter activity of GnRH fluctuated in approximately one hour period. Subsequently, fluctuation of GnRH promoter activity was shown to be functionally associated with episodic exocytic activity (Vazquea-Martinez et al., 2001). It was suggested that the fluctuation at the promoter level was also associated with intracellular calcium oscillation (Nuñez et al., 2000).

3. Kisspeptin and its role in regulating GnRH neuron

3.1. Kisspeptin-GPR54 system

It is now well established that kisspeptin, encoded by the Kiss1 gene, is a cognate ligand for GPR54, also called as KISS1R (Oakley et al., 2009). However, each component of this ligand-receptor pair was initially discovered independently. Kiss1 was initially discovered as mRNA transcript with metastasis-suppressor activity (Lee et al., 1996). This finding was followed by the cloning and chromosomal localization of Kiss1 gene (West et al., 1998). Kiss1 mRNA is translated to form a 145-amino-acid propeptide called kisspeptin-145. Cleavage leads to the production of RF-amidated kisspeptin-54. Shorter peptides, such as kisspeptin-10, -13, and -14, sharing a common C terminus with kisspeptin-54 exhibit similar biological activity shown in kisspeptin-54 (Ohtaki et al., 2001; Muir et al., 2001). On the other hand, GPR54 was discovered by the library screening utilizing degenerate PCR in attempt to find a novel G protein-coupled receptor (Lee et al., 1999). Although GPR54 shares a modest sequence homology with the galanin receptors, it does not exhibit a specific affinity to a panel of ligands, including galanin and galanin-like peptides (Ohtaki et al, 2001).

In 2001 that three independent groups identified kisspeptin as a natural ligand for GPR54 (Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001). Subsequently, the putative roles of kisspeptin-GPR54 in the control of

tumor progression were investigated (Ohtaki et al., 2001; Roa et al., 2008). Various forms of kisspeptin are able to suppress metastasis in several tumors, including melanoma, pancreatic cancer and ovarian carcinoma (Lee et al., 1997; Jiang et al., 2005; Masui et al., 2004). Additionally, down-regulation of Kiss1 expression was suggested as a diagnostic marker for a variety of tumors such as melanomas, gastric and bladder carcinomas (Lee et al., 1997; Dhar et al., 2004; Sanchez-Carbayo et al., 2003). However, the metastasis-suppressive role of kisspeptin-GPR54 was not consistent in other types of tumors, including human breast cancer and hepatocellular carcinoma (Martin et al., 2005; Schmid et al., 2007), thus raising a question of whether kisspeptin is a universal metastasis suppressor.

The role of kisspeptin-GPR54 was widely examined based on its tissue distribution (Ohtaki et al., 2001; Muir et al., 2001), suggesting its presumptive roles in the placenta, pancreatic islets, and aorta (Roa et al., 2007). Expressions of Kiss1 and GPR54 have also been found in the brain, including hypothalamus, basal ganglia, amygdala, substantia niagra, and hippocampus (Ohtaki et al., 2001; Muir et al., 2001).

3.2. Role of kisspeptin in regulation of GnRH neurons

Kisspepin and GPR54 have serendipitously emerged as key players in the regulation of GnRH neurons, and thus the reproductive system. In 2003, two groups independently reported that loss-of-function mutations in GPR54 are associated with hypogonadotropic hypogonadism (Seminara et

al., 2003; de Roux et al., 2003), which is characterized by delayed or lack of puberty primarily caused by gonadotropin deficiency. Severe reproductive dysfunction was observed in knock-out mouse model bearing targeted deletions of GPR54 (Funes et al., 2003; Seminara et al., 2003), whereas hypothalamic GnRH content was unaffected. Genetic deletion of Kiss1 consistently elicited hypogonadotropic hypogonadism (d'Anglemont de Tassigny et al., 2007) with normal GnRH neuronal population in the hypothalamus and responsiveness to exogenous kisspeptin administration. Dominant GPR54 mutation (R386P) was identified in a girl with idiopathic central precocious puberty (Teles et al., 2008). Taken together, these findings indicate that Kiss1-Gpr54 is an authentic ligand-receptor pair which plays a key role in the regulation of the reproductive system, presumably at the upstream levels of GnRH neurons.

Potent stimulatory action of kisspeptin on LH/FSH release has been shown in a variety of animal species, including mouse, monkey and human (Gottsch et al., 2004; Dhillo et al., 2005; Messager et al., 2005; Shahab et al., 2005). The stimulatory action of kisspeptin on GnRH and LH secretion was evident at different stages of postnatal development, and in both sexes (Castellano et al., 2006). Compared to previously known GnRH secretagogs, such as glutamate and galanin-like peptide, kisspeptin can evoke the GnRH/LH secretion even at a several orders of lower concentration (Roa et al., 2008). Additionally, electrophysiological approach revealed that application of kisspeptin evoked a remarkably strong and long-lasting depolarization in GnRH neuron in both male and female mice

(Han et al., 2005). Therefore, kisspeptin is currently regarded as the most potent activator of GnRH neurons functioning as the most efficient secretagogue of GnRH/LH release.

Kisspeptin-expressing neurons are mainly localized in two separate nuclei of the forebrain. One kisspeptin nucleus is found around the anterior hypothalamic regions, while exact locations slightly vary across different species – the anteroventral periventricular nucleus (AVPV) in rodents, the POA in sheep, the periventricular nucleus in pigs, monkey or rostral periventricular area and ventral periventral nucleus in human (Clarkson and Herbison, 2006; Ramaswamy et al., 2008; Hrabovszky et al., 2010). The other kisspeptin nucleus is located in the ARC in several examined species, including rodents, sheep, pig and primates. (Oakley et al., 2009). Major kisspeptin immunoreactive fibers are found within the ventral aspect of the lateral septum and along periventricular and ventral retrochiasmatic pathways as well as median eminence (Clarkson and Herbison, 2006; Ramaswamy et al., 2008). Interestingly, immunohistochemical analysis demonstrated close apposition between kisspeptin fibers and GnRH cell bodies (Clarkson and Herbison, 2006; Kinoshita et al., 2005) or GnRH fiber (Decourt et al., 2008), suggesting kisspeptin neurons may directly innervate GnRH neurons.

It is worth noting that the majority of GnRH neurons express GPR54. Using lacZ reporter expressed in GPR54 knockin mice, β-galactosidase activity is observed in approximately 55% of GnRH neuronal cell bodies located in the POA (Messager et al., 2005). Dual labeling in situ

hybridization studies revealed that more than 90% of GnRH neurons express GPR54 transcript (Han et al., 2005). Although kisspeptin may act through synaptic mechanisms to stimulate GnRH secretion, it may also act directly in a non-synaptic or paracrine manner (Oakley et al., 2009). In addition to direct action on GnRH neurons, it is also suggested that kisspeptin may act on interneurons, such as GABAergic cells, to regulate GnRH neurons (Pielecka-Fortuna and Moenter, 2010; Zhang et al., 2009).

3.3. Dynamic regulation of GnRH neuron by kisspeptin and its implication in the GnRH pulse generator

Phenotypes observed in Gpr54 mutant studies demonstrated that hypogonadotropism is not due to a developmental deficit of GnRH neurons, loss of biosynthetic capacity to synthesize GnRH in the hypothalamus nor responsiveness to gonadotropin-releasing hormone at the level of pituitary gonadotropes (Roa et al., 2008; Seminara et al., 2003; Funes et al. 2003; d'Anglemont de Tassigny et al., 2007). Overall, those observations suggested that kisspeptin-GPR54 is involved in the dynamic control of the GnRH system, whose function appeared to be shut-down in conditions of defective GPR54 signaling or absence of kisspeptin.

Considering the dynamic nature of kisspeptin control of GnRH neurons, the next question was focused on whether the efficacy of kisspeptin signaling is dependent on its biological rhythm, with an emphasis on the possible inhibitory action of kisspeptin which is analogous to suppression of

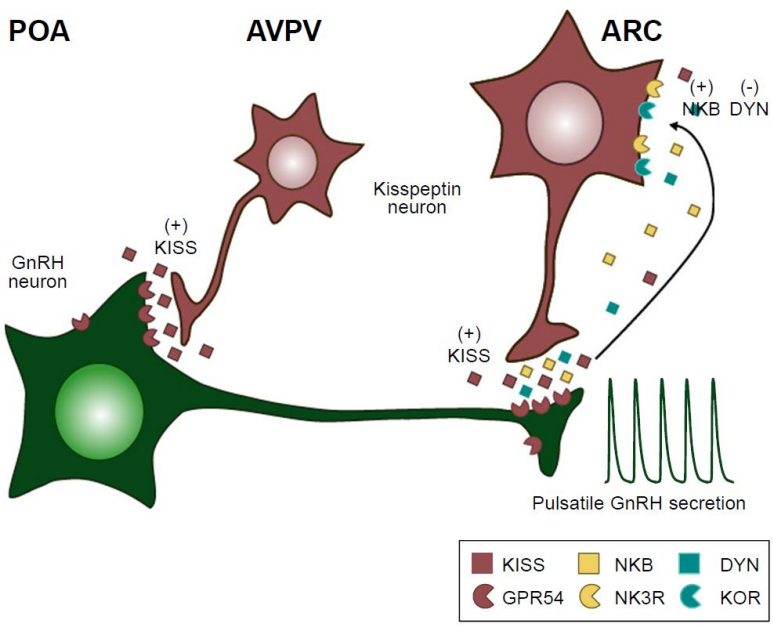
gonadotropin release evoked by chronic exposure to GnRH or its agonists. Continuous delivery of exogenous kisspeptin appears to desensitize GPR54, resulting in decreased LH secretion in agonadal juvenile and adult male monkeys and testicular degeneration in adult male rats (Seminara et al., 2006; Ramaswamy et al., 2007; Thompson et al., 2006). In contrast, repeated peripheral injections of kisspeptin elicits normal LH pulses in male rats and monkeys, implying that the efficacy of kisspeptin to drive GnRH/LH secretion depends on its pulsatile nature, similar to GnRH (Tovar et al., 2006; Plant et al., 2006).

Critical roles of kisspeptin in regulation of GnRH neurons raised a question of whether kisspeptin is involved in the generation of pulsatile GnRH release. In the stalk-median eminence of rhesus monkey, the GnRH and kisspeptin levels were determined using microdialysis technique and found to be highly correlative (Keen et al., 2008). Kisspeptin release in the stalk-ME exhibited a distinct pattern of hourly pulsatility which simultaneously reached its peak with GnRH release or slightly preceded GnRH. Development of pharmacological inhibitor facilitated the analysis of kisspeptin effect on GnRH pulse generator (Roseweir et al., 2009). Central administration of kisspeptin antagonist, PEP234, promptly abolished pulsatile GnRH release at the stalk-ME in rhesus monkey and secretory LH pulses in the ewes, while basal secretion of GnRH/LH appeared unaffected in both cases.

It is important to note that kisspeptin neurons in the ARC is tripeptidergic, co-expressing neurokinin B (NKB) and dynorphin A (DYN)

(Navarro et al., 2009; Maeda et al., 2010; Navarro, 2012). As summarized in figure 4, NKB is capable to activate the kisspeptin neurons via its receptor Nk3r (also called Tacr3), while DYN acts as a negative regulator via its receptor Kor (Lehman et al., 2010; Navarro, 2012). The feedback loop formed by positive (NKB) and negative (DYN) regulatory neuropeptide satisfies a minimal prerequisite for the

Figure 4. Schematic diagram showing GnRH neurons and kisspeptin neurons. The cell bodies of GnRH neurons are enriched in the preoptic area (POA), extending their axons to the median eminence (ME) to secret GnRH into the hypothalamic-pituitary portal vessel in a pulsatile manner. Kisseptin exerts its effect through its receptor GPR54, expressed in the GnRH neurons. Kisspeptin neurons in the arcuate nucleus (ARC) also express and secret neurokinin B (NKB) and dynorphin A (DYN), and their receptor NK3R and KOR, respectively. Autoregulatory feedbackloop formed by positive element NKB and negative element DYN may play a role in an ultradian pattern of oscillation in the level of kisspeptin.


POA
AVPV
ARC
(+)
NKB
(-)
DYN
Kisspeptin
neuron
(+)
KISS
GnRH
neuron
(+)
KISS
Pulsatile GnRH secretion
KISS
NKB
DYN
GPR54
NK3R
KOR

generation of biological rhythm. Taken together, accumulating evidences suggests that kisspeptin may be involved in the mechanism for the GnRH pulse generator.

PURPOSE

While robust ultradian rhythm in pulsatile GnRH secretion with approximately one-to-two-hour period have been recognized for decades, molecular and cellular mechanism for GnRH pulse generator remains still elusive. Although accumulating evidence suggests the presence of an intrinsic oscillator in GnRH neuron, it is still unclear the dynamics of GnRH gene expression and its contribution to the GnRH pulse generator at the level of GnRH neuronal population. Therefore, the present study aims to elucidate the following issues:

(1) How is the temporal dynamics of GnRH gene expression at the level of a single cell? And how are individual GnRH neurons synchronized to secret GnRH in a pulsatile manner?

(2) What is the role of kisspeptin in the regulation of the GnRH pulse generator?

(3) Is there hierarchical interaction between molecular circadian clock and the ultradian GnRH pulse generator?

These specific questions will be addressed in detail in the following chapters.

CHAPTER 1

Ultradian rhythm of GnRH gene expression and synchronization by kisspeptin

ABSTRACT

Pulsatile GnRH secretion is essential for pituitary gonadotrope function. Although the importance of pulsatile GnRH secretion has been well recognized for several decades, mechanism underlying GnRH pulse generation in hypothalamic neural networks remains elusive. Here, I demonstrate ultradian GnRH gene transcription in a single GnRH neuron using cultured hypothalamic slices prepared from transgenic mice expressing the GnRH promoter-driven destabilized luciferase (GnRHp-dsLuc). Under basal, unstimulated conditions, the GnRH promoter activity in each GnRH neuron exhibits an ultradian pattern of oscillations with an approximate 10-hr period. The GnRH neuronal populations in culture, however, exhibited partially synchronized bursts of GnRH transcriptional activity of ~2-hr intervals. Surprisingly, intermittent administration of kisspeptin, a potent activator of GnRH neurons, evoked a dramatic synchronization of GnRH gene transcription throughout the GnRH neurons which was accompanied by a robust stimulation of the pulsatile GnRH secretion. In conclusion, pulsatile GnRH gene expression in hypothalamic neuronal population associated with episodic GnRH secretion provides a novel insight into GnRH pulse generation.

Keywords: gonadotropin-releasing hormone (GnRH), pulse generator, ultradian rhythm, kisspeptin, gene expression, episodic secretion

INTRODUCTION

Diverse forms of biological oscillation are found in biochemical reactions, cellular events and even physiological processes (Goldbeter, 2006). Since the recent identification of the molecular circadian clock controlling intrinsic daily rhythms (Takahashi et al., 2008; Dibner et al., 2010), there have been great advances in our understanding of chronobiology in general and specifically of biological rhythms as exemplified by the continuous dynamic equilibration found in the activity of hypothalamic-pituitary-adrenal axis (Lightman and Conway-Campbell, 2010). The pulsatile release of hypothalamic neurohormones into the hypothalamic-pituitary portal vessels is a classic example of ultradian biological oscillation. In this regard, gonadotropin-releasing hormone (GnRH) has been the most extensively studied neurohormone, and its pulsatile neurosecretion has been clearly shown to be crucial for the normal functioning of the reproductive axis as elegantly demonstrated in seminal experiments by Knobil and colleagues (Knobil, 1980). Although the physiological importance of pulsatile GnRH secretion has been recognized, the cellular mechanism underlying the GnRH pulse generation remains to be elucidated.

It has been suggested that there is an autonomous mechanism for generating a pulsatile secretion within individual GnRH neurons. Pulsatile GnRH release *in vitro* has been demonstrated in immortalized GnRH-producing GT1 cells, as well as in hypothalamic GnRH neuron cultures (Martínez de la Escalera et al., 1992; Terasawa et al., 1999a). The

intracellular mechanisms of calcium oscillation (Jasoni et al., 2010), episodic gene expression (Nuñez et al., 1998) and voltage-dependent ion channel-mediated synchronization (Vazquez-Martinez et al., 2001) have all been proposed to underlie pulsatile GnRH release. Electrophysiological studies also have suggested a synchronization mechanism underlying the autonomous pulse generation (Moenter et al., 2003; Campbell et al., 2009). It has been noted, however, that most of the GnRH neuronal cell bodies are located in the POA of the hypothalamus, and direct contact between the GnRH neuronal cell bodies and dendrites are only found occasionally (Witkin et al,. 1982; Witkin and Silverman, 1985). Indeed, these anatomical and morphological features make it more plausible that the scattered GnRH neurons form neural networks and coordinate their function, presumably along with neighboring non-GnRH neurons.

The kisspeptin-producing cell bodies mainly reside in two discrete hypothalamic regions, the anteroventricular periventricular nucleus (AVPV) and the arcuate nucleus (ARC) and extend their neurite in adjacent to axonal terminal and cell bodies of GnRH neurons (Roa et al., 2008; Oakley et al., 2009). Since the first clinical observation that mutations in the kisspeptin receptor (GPR54) are associated with hypogonadotropic hypogonadism (Seminara et al., 2003), roles of kisspeptin in control of GnRH neuron are established in the multiple processes including pubertal maturation and metabolic control (Roa et al., 2008; Oakley et al., 2009; Navarro and Tena-Sempere, 2011). In this regard, kisspeptin is proposed to participate in the generation of GnRH pulse as hypothalamic components

(Li et al., 2009; Roseweir et al., 2009), while the mechanism still remains elusive. I designed the present study, therefore, to elucidate the episodic pattern of GnRH gene expression and secretion in the GnRH neuronal population and neural circuits of the hypothalamus using organotypic culture model.

MATERIALS AND METHODS

Animal care and handling. C57BL/6J mice were housed in temperature-controlled (22~23°C) quarters under a 12-hr light and 12-hr dark (LD) photoperiod (light-on at 8:00 a.m.); standard mouse chow and water were available *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

GnRHp-dsLuc plasmid. Luciferase gene fragments were amplified from pGL3-basic (Promega, Madison, WI) by PCR using a forward primer containing the HindIII restriction site and a reverse primer with the XbaI and EcoRI restriction sites. The luciferase gene of pGL3-basic was replaced with the PCR product. The PEST sequence was derived from mouse ornithine decarboxylase (MODC) ubiquitination fragments. The PCR product containing the PEST sequence was tagged to the luciferase gene using the EcoRI/XbaI sites to construct destabilized luciferase. The rat gonadotropin-releasing hormone (GnRH) promoter (3.0 kb, -3002 to +88 from the transcription start site) was constructed from the plasmid pGnRH3.0-Luc (Cho et al., 1998). Using the XhoI/HindIII sites, the promoter was cloned into the modified pGL3-basic vector. The primer sequences used for cloning were as follows: Luciferase forward, 5'- AAG CTT AAG CCA CCA TGG AAG ACG CCA-3'; Luciferase reverse, 5'-TCT AGA GGG GAA TTC CAC GGC GAC TTT CCG CCC TT-3'; PEST forward, 5'-GAA TTC AGC CAT GGC TTC CCG CCG GCG GTG GCG GCG CAG

GAT GAT-3'; and PEST reverse, 5'-TCT AGA CTA AAG CAG GTC CTC CTC TGA GAT CAG CTT CTG CAT TGA TGC CAT CAC ATT GAT CCT AGC A-3'.

Generation of GnRHp-dsLuc transgenic (TG) mice. TG mice were generated as described previously (Son et al., 2008). Briefly, the inserted fragment containing the GnRH promoter, destabilized luciferase, and SV40 poly-A signal was cut out by XhoI/XbaI digestion and purified by agarose gel electrophoresis. The TG mice were generated by microinjection of the purified DNA into the pronuclei of the fertilized eggs of C57BL/6J mice. The animal genotypes were identified by PCR amplification of tail DNA. PCR genotyping was carried out with the following two primers: GnRHp-dsLuc forward for genotyping, 5'-GTG GCT TCA GCT GTG AAA GT-3'; GnRHp-dsLuc reverse for genotyping, 5'-CAC CTC GAT ATG TGC ATC T-3'. The tissue distribution of luciferase activities was determined with a commercial enzyme assay kit (Promega) and normalized with a protein assay kit (BioRad, Hercules, CA) according to the manufacturer's instructions.

Immunohistochemistry. Male GnRHp-dsLuc mice (15 weeks of age) were perfused with 4% paraformaldehyde in PBS, and the brains were post-fixed in the same solution for 24 h. The brains were then cryoprotected in 30% sucrose and sectioned (20 μm); subsequent immunostaining was performed using a free-floating method. Brain sections containing POA were washed with PBS and blocked with 3% BSA and 0.1% Triton X-100 in

PBS for 30 min. The following primary antibodies were applied overnight at 4°C: anti-GnRH (Millipore, Billerica, MA), 1:500; anti-Luciferase (Sigma, St. Louis, MO), 1:500. After three washes with PBS, the appropriate secondary antibodies conjugated with fluorescent dye were applied for 30 min. Subsequently, the sections were washed, mounted, and observed under fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

Slice culture. The materials for slice culture were obtained from Invitrogen (Carlsbad, CA). The slice culture was prepared as previously reported with minor modifications (Yamaguchi et al., 2003). Briefly, the neonate genotype was determined on postnatal day 3-5 by PCR from tail genomic DNA. Transgenic mice (postnatal day 5-7) were anesthetized with ether, and the removed brains were immediately transferred to ice-cold Gey's balanced salt solution (GBSS; with 10 mM HEPES, 30 mM glucose) bubbled with 5% CO_2 and 95% O_2. The coronal or sagittal brain slices (400-μm thick) were made with a vibratome (Campden Instruments, Leicester, UK) and the brain slices were carefully dissected under a stereomicroscope to minimize the outside regions of the preoptic area (POA), which located at 3.10 to 3.50 mm according to the Paxinos coordinate description (postnatal day 6) (Paxinos et al., 2003). The POA region was clearly distinguished from the suprachiasmatic nucleus, located at 4.10 to 4.60 mm, and the arcuate nucleus, 4.80 to 6.00 mm. For the adult slices, brain slices containing the POA were sectioned at 200 μm using a vibratome. The POA explants (approximately 1 mm long and 1 mm wide) were maintained on a

membrane (Millicell-CM, Millipore) dipped into a culture medium (50% minimum essential medium, 25% Hank's balanced salt solution, 25% horse serum, 36 mM glucose, and 100 U/ml penicillin-streptomycin) at 36°C. For the experiments which included the measurement of GnRH secretion, POA slices from four neonatal mice were maintained on a single membrane. The medium was changed every three days by lifting the Millicell culture membrane and placing it into a new culture dish containing fresh medium.

Drugs. The drug concentrations were as follows: 15a (Kobayashi et al., 2010) (4Chem, Seoul, Korea) 30 μM; Gö 6983 (Tocris, Bristol, UK) 10 μM; cycloheximide (Sigma) 100 μM; brefeldin A (BFA, Sigma) 10 μg/ml; tetrodotoxin (TTX, Tocris) 0.5 μM; nimodipine (Nimo, Tocris) 10 μM. Slices were incubated with each drug for at least 2 hrs before kisspeptin administration.

Single-cell real-time bioluminescence monitoring. The bioluminescence from the POA cultures was monitored as previously reported with slight modifications (Yamaguchi et al., 2003). The bioluminescence from the POA cultures was monitored with a Cellgraph (AB-1000, ATTO, Tokyo, Japan), a specialized microscope for bioluminescence observation, equipped with a Nikon S Fluor 10X objective lens (NA, 0.75). The POA slice cultures were maintained in a customized chamber (Live Cell Instruments, Seoul, Korea) and kept in an incubating unit inside the Cellgraph (36°C, 5% CO_2 balanced with air and humidified). One millimolar D-luciferin (Promega) was included

in the recording medium (DMEM:F12 supplemented with 1X N2 supplement, 36 mM glucose, 100 U/ml penicillin-streptomycin). Images of 4 min exposure duration were acquired at 5 min intervals using the Cellgraph Controller (ATTO). The image acquisition conditions were as follows: Cooling temperature: -80°C; Binning: 1X1; Readout rate: 1 MHz at 16-bit; Output amplifier: electron multiplying; Electron multiplier gain: 200; and Pre-amplifier gain: 1.0. The acquired bioluminescence images were analyzed using MetaVue (Molecular Devices, Sunnyvale, CA). All of the analyses were restricted to cells that were clearly discriminable from adjacent cells. Luminescence intensity was measured within a region of interest defined manually for each cell and filtered through a median filter to eliminate cosmic-ray-induced noise. Average value of the region of interest was presented as arbitrary unit (A.U.) after background correction. The detrended value was obtained by subtracting the 25-min average from the background-eliminated luminescence intensity.

Intermittent kisspeptin stimulation and measurement of the hormone level. For the simultaneous determination of GnRH secretion with bioluminescence monitoring, the POA cultures were maintained in a customized chamber with two input ports and one output port made of stainless steel. Two NE-1000 syringe pumps controlled by a Syringe Pump Pro program (New Era Pump Systems, Farmingdale, NY) were connected with each of the input ports (void volume, approximately 200 μl). The media in the chamber was withdrawn using a Minipuls Evolution peristaltic pump

(Gilson, Middleton, WI) connected with the output port (void volume, approximately 300 μl). For intermittent kisspeptin administration, 6 consecutive kisspeptin pulses (15-min kisspeptin (10 nM) followed by 45-min media washout) were administered to the slice culture. The recording media was perfused at a flow rate of 2.4 ml/hr and collected every 15 min. The collected media was centrifuged at 12,000 rpm for 3 min and stored at -80°C until radioimmunoassay (RIA). GnRH RIA was performed using Chen–Ramirez GnRH antiserum, CRR13B73 (generously provided by V. D. Ramirez, University of Illinois, IL). The sensitivity at 90% binding was approximately 5 pg/tube. The intra- and inter-assay coefficients of variation were 4–5 and 5–10% for a 10 pg dose of synthetic GnRH, respectively.

Single-cell real-time monitoring data analysis. The pulse peak and nadir of the background-subtracted bioluminescence profile were identified using the Cluster-8 program (Veldhuis and Johnson, 1986). Cluster sizes for peaks and nadir were defined as 3, and the *t* statistics to identify a significant increase or decrease was 2.0. The pulse period of an individual cell was defined as the duration between the first and last peak divided by the number of interpulse intervals. The pulse amplitude was normalized by the average bioluminescence activity. The synchronization of a GnRH neuronal population was calculated as previously described (Moore et al., 2002) with minor modifications. Briefly, the time points when the peaks were detected in each cell were compiled into a single file for the entire neuronal population within the imaged field. The EXCEL program assigned

either a 0 (no significant peak) or 1 (significant peak detected) for a given point. This series of 0s and 1s was summed and normalized by the number of cells detected in the culture.

Heatmap plot. For the heatmap plots, detrended bioluminescence intensity data were normalized by standard deviation and then color coded for a higher than 1.5 standard deviation in red, and a lower than 1.5 standard deviation in green. Plots were constructed using HCE3.5 (Seo et al., 2006).

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Statistical significance was assessed by unpaired two-tailed Student's *t*-test or two-way ANOVA. Bonferroni post-test was used for *post hoc* comparison of ANOVA. Statistical significance was set as follows: *, $P<0.05$; **, $P<0.01$.

RESULTS

Ultradian oscillation of GnRH promoter activity revealed by real-time bioluminescence imaging of individual hypothalamic GnRH neurons

For real-time monitoring of GnRH gene transcription, I used transgenic mice bearing a destabilized luciferase reporter under the control of the 3.0 kb rat GnRH promoter (GnRHp-dsLuc) (Fig. 5A). I compared the expression profiles of the luciferase reporter with those of endogenous GnRH to validate the model. In the preoptic area (POA), most luciferase-immunoreactive neurons co-expressed the GnRH decapeptide and the relative luciferase activities correlated well with the GnRH content in several of the examined tissues; the strongest transgene expression was found in the POA, and considerable expression was also found in the olfactory bulb and hippocampus (Fig. 5B-D). Moreover, GnRH promoter-driven luciferase activity exhibited similar changes with the GnRH content across the estrous cycle (Fig. 5E, F). These observations clearly indicate that the luciferase expression in these GnRHp-dsLuc transgenic mice closely parallel the spatio-temporal regulation of endogenous GnRH biosynthesis.

I prepared organotypic slice cultures using the POA of the transgenic animals on postnatal days 5-7 (Fig. 6A), which were then subjected to real-time bioluminescence imaging after 2-4 weeks of maturation (Fig. 6B). By using the system, I can assess the GnRH promoter activity of a single

GnRH neurons in the hypothalamic preparation in real-time.

The POA slice culture exhibited scattered distribution of GnRH neurons (Fig. 7A left panel), which is typical in the hypothalamic GnRH neuronal distribution (Herbison, 2006). 20-30 bioluminescence-positive cells were simultaneously monitored in a POA slice culture. Single-cell real-time bioluminescence analysis revealed that the GnRH promoter activity in an individual neuron exhibited irregular but distinct ultradian bouts (Fig. 7A right panels). Pseudocolor-coded time lapse images of a single GnRH neurons clearly demonstrated ups and downs in bioluminescence intensity which reaches their peak approximately twice to three times a day. Quantified profiles of single GnRH neuron also demonstrated the ultradian oscillatory pattern of GnRH promoter activity (Fig. 7B).

Due to irregular nature in the oscillation of GnRH gene transcription, I monitored more than a hundred GnRH neurons and found a consistent ultradian oscillation throughout the analyzed cells (Fig. 8A). Although individual interpulse period varies from peaks to peaks, statistical analysis revealed that the mean interpulse interval was approximately 10 hrs (594.51 ± 13.49 min, n=124 from 5 slices; Fig. 8B) and the amplitude was 105.12 ± 4.66% of the average bioluminescence (n=124 from 5 slices; Fig. 8C).

Ultradian oscillation of GnRH promoter-driven luciferase expression in the GnRH neurons under various preparation conditions

Even though the cell bodies of GnRH neurons are mostly enriched in the POA regions, the GnRH neurons are also present in the diagonal band of Broca (DBB) which is directly rostral to the POA (Wray, 2010). To examine the dynamics of GnRH promoter activity in the DBB GnRH neurons, I prepared organotypic slices derived from DBB region of GnRHp-dsLuc transgenic mice. Similar to GnRH neurons in cultured POA slices, ultradian bouts of GnRH promoter activities were observed in GnRH neurons in the slice cultures of the DBB (Fig. 9A, B). Average period of bouts of GnRH promoter activities were slightly shorter in GnRH neurons in DBB compared with those in POA (Fig. 9C, 592.06 ± 21.77 min for POA vs. 549.98 ± 15.11 min for DBB, P = 0.1153), although it was not statistically significant. The differences in average amplitude of ultradian profiles were rarely different between two groups (Fig. 9D, 96.36 ± 7.10% for POA and 95.62 ± 5.49% for DBB, P = 0.9350, n = 52-55 cells from 3 slices for each group).

Morphological analysis utilizing neurobiotin suggested that GnRH neurons may interact through dendritic contact among GnRH neurons themselves, which is more frequently in coronal direction compared with sagittal direction (Campbel et al., 2009). In this regards, I compared the spontaneous ultradian bouts of GnRH transcription in neurons derived from either coronal or sagittal section (Fig. 10A, B). In the sagittal slice, the expression of GnRH promoter-driven luciferase was episodic in the sagittal slice with approximately 10-hr interval (Fig. 9A and B, 654.31 ± 26.13 min, n = 27 from 2 slices), similar to the coronal slice. The GnRH neurons in the

sagittal slices exhibited ultradian oscillation with the lower amplitude than those in the coronal slices (Fig. 10C, D).

Preparation age on the spontaneous ultradian oscillation of GnRH promoter activity

During *ex vivo* cultivation after slice preparation, organotypic primary culture undergoes maturation process including thinning and glial proliferation (Stoppini et al., 199). Hypothalamic content of GnRH gradually increase to reach an adult level at the puberty during postnatal development. Therefore following set of experiments are designed to examine the influence of age at slice preparation and *ex vivo* maturation on temporal pattern of GnRH transcription.

First, I compared real-time profiles of GnRHp-dsLuc bioluminescence either in slices which have matured for ~ 2 weeks or acutely prepared slices. Consistently with cultured slices (Fig. 11A and B, upper panels), GnRH neurons in acutely prepared slices exhibited ultradian pattern of luciferase activities (Fig. 11A and B, middle panels). Ultradian bouts of bioluminescence in acutely prepared slice were also quite irregular but episodic.

Next, I monitored GnRH transcription in hypothalamic slice acutely prepared from adult GnRHp-dsLuc transgenic mice. Even the POA cultures acutely prepared from the adult transgenic mice exhibited ultradian fluctuation in GnRH promoter activity, which reached its peak twice or three

times a day (Fig. 11A and B, lower panels). Limited *ex vivo* viability of GnRH neurons derived from adult animals prevented the real-time monitoring of cultured adult hypothalamic slices.

Key features of ultradian oscillation in GnRH transcription were relatively consistent regardless of preparation age or *ex vivo* cultivation. The average period of ultradian oscillations was approximately 10 hours in cultured neonatal slices (CN), acutely prepared neonatal slices (AN), and acutely prepared adult slices (AA) (Fig. 11C). In all three of examined conditions, the amplitude of ultradian oscillations was approximately same as their average intensity over incubation time (Fig. 11D). Considering the consistent ultradian characteristics in GnRH transcription observed in various preparations, it is considered to be a persistent property of postmitotic GnRH neurons.

Spontaneous oscillation is cell-autonomous

Next set of experiments were designed to examine the source of ultradian bouts in GnRH gene transcription. To evaluate the influence of the neighboring neuronal input, synaptic input was blocked by application of tetrodotoxin (TTX), a sodium channel blocker. After 1 day of pre-treatment session, 2 days of real-time monitoring was followed in the presence of TTX (1 μM). Application of TTX did not elicit any apparent effect in the ultradian GnRH transcription under basal conditions (Fig. 12A, upper and middle panels). The average pulse period slightly decreased after TTX applications

(Fig, 12B), the pulsatile amplitude of luciferase activities appeared to be unaffected (Fig.12C).

Oscillation of intracellular calcium level is one of the prominent biological rhythms in GnRH neuron (Jasoni et al., 2010). In GT1 cell line model, GnRH transcription and calcium influx through L-type calcium channel was suggested to be linked because blockage of L-type calcium channel by using nimodipine (Nimo) led to decline of GnRH transcription (Vazquez-Martinez et al., 2001). Therefore, I monitored spontaneous bioluminescence emitted from GnRH under basal contiditions followed by application of nimodipine (10 μM). Spontaneous oscillation of bioluminescence driven by GnRH promoter persisted in the presence of nimodipine (Fig. 12A, bottom panels). Average period and amplitude was rarely affected by application of nimodipine (Fig. 12B). In contrast to GT1 cell line, ultradian rhythm of GnRH transcription in postmitotic GnRH neurons in organotypic hypothalamus culture does not appear to rely on L-type calcium channel.

These results indicate that ultradian GnRH gene transcription occurring with an approximate 10-hr period appears to be an intrinsic feature of postmitotic GnRH neurons. Persistent ultradian oscillation in the presence of TTX and nimodipine indicates that the ultradian rhythmicity of GnRH transcription is cell-intrinsic and based on a yet unidentified mechanism.

Synchronization of GnRH neurons under basal conditions

It is noteworthy that the 10-hr interpulse interval found in the pattern of GnRH gene transcription is relatively longer than that of endogenous episodic neurohormone secretion, which is reported to be 1-2 hr (Knobil, 1980; Herbison, 2006; Maeda et al., 2010). Since the pulsatile GnRH release from the hypothalamus is a result of the coordinated discharge of the decapeptide from the GnRH neuronal population, I compared the simultaneously occurring peaks of the ultradian GnRH promoter activity as a measure of the synchronization of the individual cells in a given cultured brain slice (typically 20 to 40 luciferase-expressing cells). Interestingly, I found that the episodes of the GnRH promoter activity exhibited a stochastically synchronized profile; a small, but significant subset of GnRH neurons (approximately 10~20% of cells) simultaneously reached a peak, forming synchronization peaks with ~2-hr intervals (118 ± 21 min from 6 independent experiments; Fig. 13A). Concomitant determination of secreted GnRH in the perifused media revealed that the synchronization of spontaneous episodes of GnRH promoter activity was only partly associated with pulsatile GnRH secretion (Fig. 13B). Although episodic GnRH gene expression in a single GnRH neuron exhibits a longer interpulse interval, this finding strongly suggests that hypothalamic GnRH gene expression *in vivo* takes place as a result of the coordination of subset of GnRH neurons and can follow an oscillation pattern with a shorter period, which is similar to that found in the pulsatile GnRH secretion from the median eminence.

Synchronization of GnRH pulse generator by pulses of kisspeptin

Kisspeptin, a neuropeptide encoded by *Kiss1* gene, is one of the strongest activators of GnRH release (Roa et al., 2008; Oakley et al., 2009). Although inhibition of kisspeptin signaling modulated the pulsatile GnRH secretion in several species (Li et al., 2009; Roseweir et al., 2009), the role of kisspeptin in the GnRH pulse generation remains elusive. I therefore examined the effects of kisspeptin pulses on ultradian GnRH gene transcription in individual GnRH neurons and their synchronization, as well as the evoked secretion of the decapeptide.

I treated POA cultures in a pulsatile fashion with kisspeptin-10, the physiologically active form (Ohtaki et al., 2001), and simultaneously analyzed the GnRH promoter activity and the associated GnRH secretion into the perifused medium. Brief administration of kisspeptin (15 min) to POA slice cultures elicited a transient increase of GnRH promoter activity in a subset of neurons (Fig. 14A and B left panel), while rest of neurons remained unaffected. Approximately 40% of GnRH neurons responded to a pulse of kisspeptin to yield brief synchronization among GnRH neuronal population (Fig 14C left panel). Contrast to a circadian oscillator which can synchronize to a single stimulation of resetting cue to generate cycles of circadian rhythm in a population level, only single transient activation in GnRH gene transcription occurred following a single pulse of kisspeptin.

Kisspeptin levels in the hypothalamus are reported to be episodic as revealed by push-pull perfusion technique (Keen et al., 2008). Therefore I

hypothesized that pulses of kisspeptin may generate the hourly pulsatility of GnRH neurons. To prove the hypothesis, I administered kisspeptin in a intermittent manner (15-min on: 45-min off, 6 consecutive pulses). GnRH promoter activities in >40% of the individual luciferase-positive cells were immediately induced in response to each kisspeptin pulse with gradual decrease in the baseline level (Fig. 14A and B, middle panels). On the other hand, approximately 30-40% of the cells exhibited a delayed response, requiring 2-3 pulses of kisspeptin to initiate transient increases in luciferase expression, illustrating the heterogeneity of GnRH neurons in their responsiveness to kisspeptin (Fig. 14C middle panel). The pulsatile treatment with kisspeptin reinforced the synchronization of GnRH gene transcription in terms of both the synchronization index and heatmap plot of the detrended pattern of luciferase activities.

Next I tried to examine whether temporal pattern of kisspeptin application is critical in its synchronizing effect. In order to apply the same amount of kisspeptin during treatment session, a pulse of kisspeptin was 10 nM (15-min on: 45-min off), while the dose of kisspeptin was 2.5 nanomolar when continuously administered (n = 3-4 batches for each scheme). In response to continuous kisspeptin stimulation, GnRH promoter activities gradually increase in a subset of neurons followed by gradual decrease (Fig. 14A and B right panel). In addition, bioluminescence signal were induced right after the termination of kisspeptin administration in a sort of GnRH neurons. The peak of promoter activity distributed rather broad range after the initiation of kisspeptin administration. Therefore the

synchronization index was not as high as in single pulse or repeated pulse stimulation (Fig. 14C right panel).

In keeping with this finding, the kisspeptin-stimulated pulsatility was also evident in secretion of GnRH. A single pulse of kisspeptin dramatically reinforced a single peak of GnRH secretion with a distinct peak approximately 30 min after the beginning of a kisspeptin pulse (Fig. 15A). While pulsatile pattern of GnRH secretion elicited by intermittent kisspeptin stimulation (Fig. 15B), GnRH secretion was gradually increased and damped by continuous kisspeptin (Fig. 15C). The peak concentration of GnRH secretion was relatively higher in a intermittent stimulation. In addition to synchronization of GnRH promoter activity, temporal pattern of kisspeptin plays a role in pulsatile activation of GnRH neurons. These results suggest that kisspeptin may be a crucial pacemaker in GnRH pulse generator, rather constitutive activator for GnRH neuron.

Specificity of kisspeptin in the generation of GnRH pulsatility

Kisspeptin neurons in the arcuate nucleus (ARC) co-express neurokinin B (NKB) and dynorphin A (DYN). Therefore co-secretion of NKB/DYN with kisspeptin may play a physiological role in the control of GnRH pulse generator. In fact, NKB is a positive regulatory element, stimulating LH secretion and accelerating the multiunit activity (MUA) (Navarro, 2012). To examine whether pulsatile application of NKB can elicit synchronization of subset of GnRH neurons, I applied pulses of senktide (SENK), a NKB

agonist, or DYN to cultured POA slice. In contrast to kisspeptin (Fig. 16A and B left panel), SENK or DYN application did not affect ultradian bouts of GnRH promoter activity (Fig. 16A and B, middle and right panel) (n = 4-5 batches for each treatment). As a result, GnRH neuronal population only exhibited basal level of synchronization following SENK pulses in contrast to pulsatile synchronization elicited by kisspeptin pulses (Fig. 16C).

In agreement with promoter activity, kisspeptin pulses dramatically induced pulsatile secretion (Fig. 17A), while SENK or DYN pulses did not reinforced pulsatile secretion of GnRH in the perifused media (Fig. 17B, C). In conclusion, the effect of kisspeptin in the generation of GnRH pulsatility is specific in comparison with co-expressed neuropeptide, neurokinin B and dynorphin A.

GPR54-PKC pathway in kisspeptin-mediated synchronization of GnRH pulse generator

Kisspeptin appears to exert effects through activation of GPR54 expressed on GnRH neurons (Roa et al., 2008; Oakley et al., 2009). To demonstrate the specificity of the kisspeptin-GPR54 interaction, I pre-treated 2-acylamino-4,6-diphenylpyridine derivative (15a), a GPR54 antagonist (Kobayashi et al., 2010), 2 hrs prior to the intermittent administration of kisspeptin. The kisspeptin effect on luciferase expression was significantly attenuated by pre-treatment with 15a, thereby suppressing the synchronization of GnRH neurons (Fig. 18A, B and 18B; $F_{(1,24)}$ = 68.47,

$P < 0.0001$ for treatment groups; $F_{(5,24)} = 2.735$, $P = 0.043$ for number of pulses; $F_{(5,24)} = 8.942$, $P < 0.0001$ for treatment-number of pulses interaction). The inhibition of GPR54 by 15a also significantly suppressed the pulsatile secretion of GnRH (Fig. 18A and C; $F_{(1,24)} = 80.17$, $P < 0.0001$ for treatment groups; $F_{(5,24)} = 3.943$, $P = 0.0094$ for number for pulses; $F_{(5,24)} = 3.335$, $P = 0.0199$ for treatment-number of pulses interaction), in addition to total GnRH secretion during kisspeptin application session (Fig. 19D).

GPR54 belongs to the G protein-coupled receptor superfamily and mainly propagates signals by the Gq protein-initiated pathway (Ohtaki et al., 2001). To elucidate the contribution of PKC pathway, 6 pulses of kisspeptin was applied to POA slice in the presence of Gö 6983, a PKC inhibitor. Pre-treatment with Gö 6983 suppressed the transient induction of luciferase activity by kisspeptin pulses (Fig. 18A and B, right panel) as well as synchronization of GnRH neurons (Fig. 18C, right panel, and Fig. 19B; $F_{(1,24)} = 148.8$, $P < 0.0001$ for treatment groups; $F_{(5,24)} = 6.061$, $P = 0.0009$ for number of pulses; $F_{(5,24)} = 7.246$, $P = 0.0003$ for treatment-number of pulses interaction). Kisspeptin-induced pulsatile GnRH secretion was significantly attenuated in the presence of Gö 6983 (Fig. 19A and C, c; $F_{(1,24)} = 90.57$, $P < 0.0001$ for treatment groups; $F_{(5,24)} = 5.628$, $P = 0.0014$ for number of pulses; $F_{(5,24)} = 1.358$, $P = 0.2749$ for treatment-number of pulse interaction). The cumulative GnRH secretion elicited by kisspeptin also decreased by pre-treatment with Gö 6983 (Fig. 19D). These findings are consistent with the fact that kisspeptin activates GPR54 and subsequent signaling pathway, implying that the kisspeptin-GPR54 system

sends potent stimulatory and synchronizing signals for both episodic GnRH gene transcription and secretory pulsatility.

De novo protein synthesis and exocytosis in the synchronized GnRH pulse generator.

The concomitant activation of episodic GnRH gene transcription and secretion raises the question of whether these processes are directly related. To address this question, I inhibited *de novo* protein synthesis or exocytosis during pulsatile applications of kisspeptin. Although previous report has claimed that the spontaneous pulsatility in GnRH release does not require gene expression or *de novo* protein synthesis (Pitts et al., 2001), 4-hr of pretreatment with cycloheximide was sufficient to block GnRH release as well as episodic reporter expression (Fig. 20A and B middle panel). The episodic neurohormone release by treatment with kisspeptin apparently disappeared in the presence of cycloheximide (Fig. 21A, C; $F_{(1,41)}$ = 19.74, P < 0.0001 for treatment groups; $F_{(5,41)}$ = 2.528, P = 0.0439 for number of pulses; $F_{(5,41)}$ = 0.5633, P = 0.7275 for treatment-number of pulses interaction) in addition to a significant reduction in cumulative GnRH secretion (Fig. 21D). Thus, it appears that GnRH biosynthesis is required to maintain kisspeptin-evoked pulsatile GnRH release, which needs to be seen in the light of previous reports that kisspeptin exerts a robust discharge effect on GnRH neurons (Messager et al., 2005).

On the other hand, exocytic activities appear to play an important role in

maintaining the synchronized responsiveness in GnRH gene transcription to tonic kisspeptinergic inputs. An inhibition of exocytosis by the application of Brefeldin A (BFA) for 4 hrs led to a gradual attenuation in episodic GnRH promoter activity induced by kisspeptin treatment (Fig. 20A, B, right panels) as well as synchronized bursts of GnRH gene transcription (Fig. 20C and Fig. 21B; $F_{(1,36)} = 49.70$, $P < 0.0001$ for treatment groups; $F_{(5,36)} = 4.644$, $P = 0.0023$ for number of pulses; $F_{(5,36)} = 2.395$, $P = 0.0566$ for treatment-number of pulse interaction). The neurohormone release was significantly impaired in the presence of the exocytosis inhibitor (Fig. 20 A, C). These results suggest that a certain releasable factor, perhaps including the secreted GnRH decapeptide itself, mediates the synchronized responses of the GnRH neurons to kisspeptin so as to coordinate the episodic GnRH gene transcription and secretion.

Figure 5. Validation of GnRHp-dsLuc transgenic mouse. (A) The structure of the GnRHp-dsLuc fragment and the position of the genotyping primer. The arrowheads indicate the primers used for genotyping. (B) Immuno-histochemical analysis of the POA region of an adult transgenic mouse. Luciferase (green) was simultaneously labeled with GnRH (red). Nucleus was counterstained with 4',6'-diamino-2-phenylindole (DAPI). More than 90% of the luciferase-positive cells were GnRH-immunoreactive. Consistent colocalization was observed in three independent batches. (C, D) Tissue distribution of endogenous GnRH peptide (C) and luciferase reporter activity (D). (E, F) Regulation of endogenous GnRH content (E) and luciferase reporter activity (F) across the estrous cycle (n = 4-6 for each group). Data are presented as the mean ± s.e.m. *: $P < 0.05$ vs. DE by two-tailed t-test.

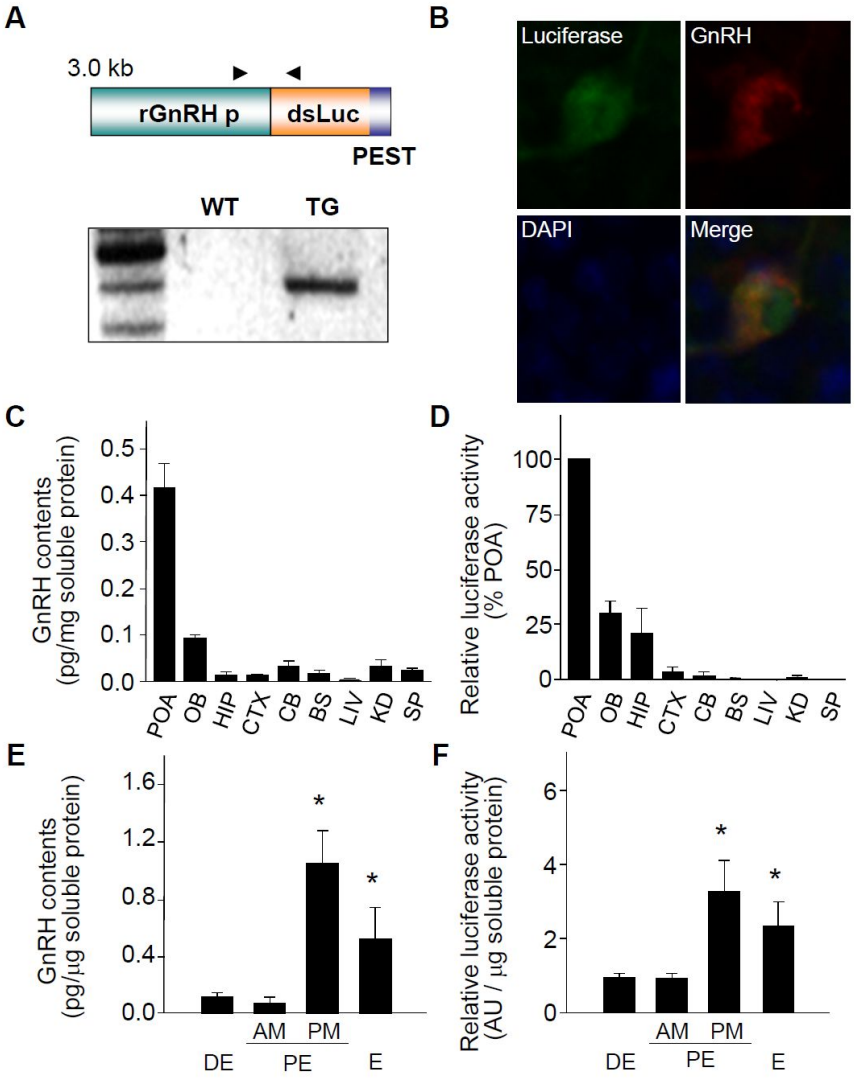

A
3.0 kb
rGnRH p
dsLuc
PEST
WT
TG
B
Luciferase
GnRH
DAPI
Merge
C
GnRH contents
(pg/mg soluble protein)
0.5
0.4
0.3
0.2
0.1
0.0
POA
OB
HIP
CTX
CB
BS
LIV
KD
SP
D
Relative luciferase activity
(% POA)
100
75
50
25
0
POA
OB
HIP
CTX
CB
BS
LIV
KD
SP
E
GnRH contents
(pg/μg soluble protein)
1.6
1.2
0.8
0.4
0.0
*
*
AM
PM
DE
PE
E
F
Relative luciferase activity
(AU / μg soluble protein)
6
4
2
0
*
*
AM
PM
DE
PE
E

Figure 6. Experimental scheme. (A) Preparation of POA slice culture. (B) Real-time bioluminescence monitoring system with a perifusion device.

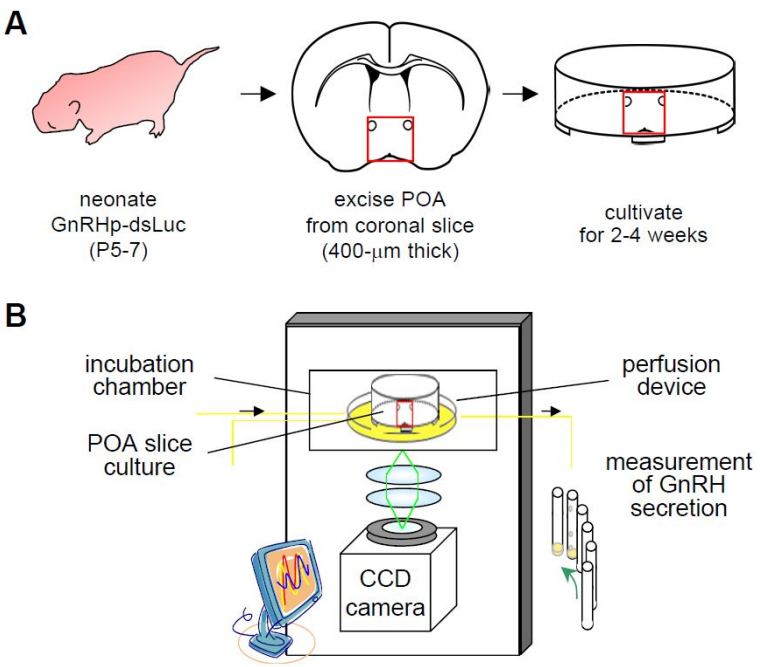

A
neonate
GnRHp-dsLuc
(P5-7)
excise POA
from coronal slice
(400-μm thick)
cultivate
for 2-4 weeks
B
incubation
chamber
POA slice
culture
perfusion
device
measurement
of GnRH
secretion
CCD
camera

Figure 7. Time-lapse single cell bioluminescence images of GnRH gene expression. (A) Bioluminescence images of POA slice culture derived from GnRHp-dsLuc mouse. Intensity of bioluminescence is color-coded according to the pseudocolor scale given beneath. Representative time-lapse images of a single GnRH neuron are shown on the right. (B) Quantitative luminescence profile of a GnRH neuron shown in (A). Red asterisks indicate the peaks identified by Cluster-8.

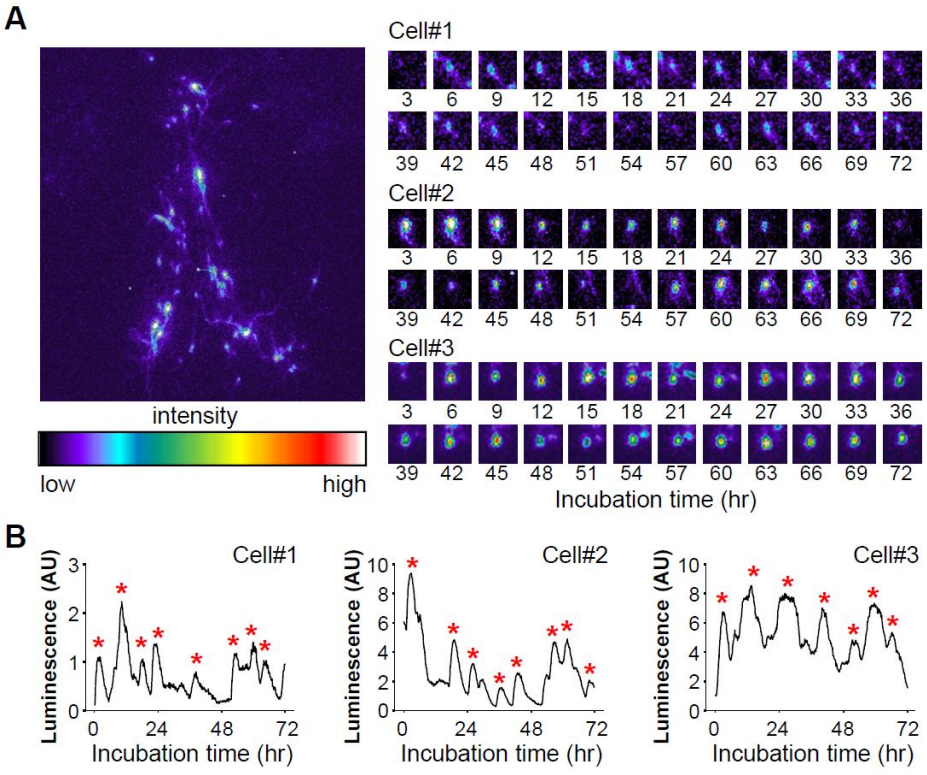

A
Cell#1
3 6 9 12 15 18 21 24 27 30 33 36
39 42 45 48 51 54 57 60 63 66 69 72
Cell#2
3 6 9 12 15 18 21 24 27 30 33 36
39 42 45 48 51 54 57 60 63 66 69 72
Cell#3
3 6 9 12 15 18 21 24 27 30 33 36
39 42 45 48 51 54 57 60 63 66 69 72
intensity
low
high
Incubation time (hr)
B
Cell#1
Cell#2
Cell#3
Luminescence (AU)
Incubation time (hr)
0 24 48 72

Figure 8. Ultradian rhythm of GnRH gene expression under basal conditions. (A) Quantitative profiles of bioluminescence determined in single GnRH neurons are shown. Red asterisks indicate the peaks identified by Cluster-8. (B, C) Stastical analysis of ultradian rhythmicity. Pulse period (B) and amplitude (C) are presented as box plot (midline, median; box, 75% and 25%; error bar, 90% and 10% percentile) (n = 220 cells from 6 batches).

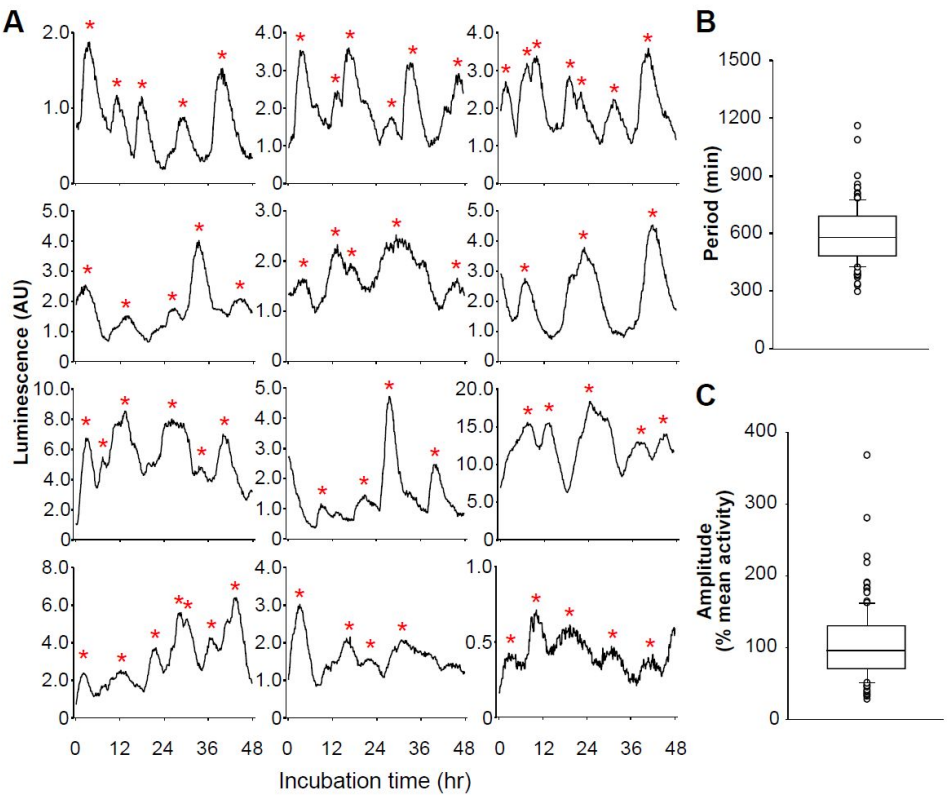

A
B
C
Luminescence (AU)
Incubation time (hr)
Period (min)
Amplitude
(% mean activity)

Figure 9. Ultradian rhythm of GnRH promoter activities in the POA and DBB. (A) Time lapse luminescence images of a single GnRH neuron (uppse, POA; lower, DBB) (B) Representative bioluminescence profiles of GnRH promoter activity in the cultures of POA (upper panels) and the diagonal band of Broca (DBB, lower panels). (C, D) Peak period (C) and peak amplitude (D) of the GnRH promoter activity observed in GnRH neurons in the POA and DBB were compared (n = 52-55 cells from 2 batches). In the box plots (C, D), data are expressed as median (line), interquartile (box), 10% and 90% percentiles (bar).

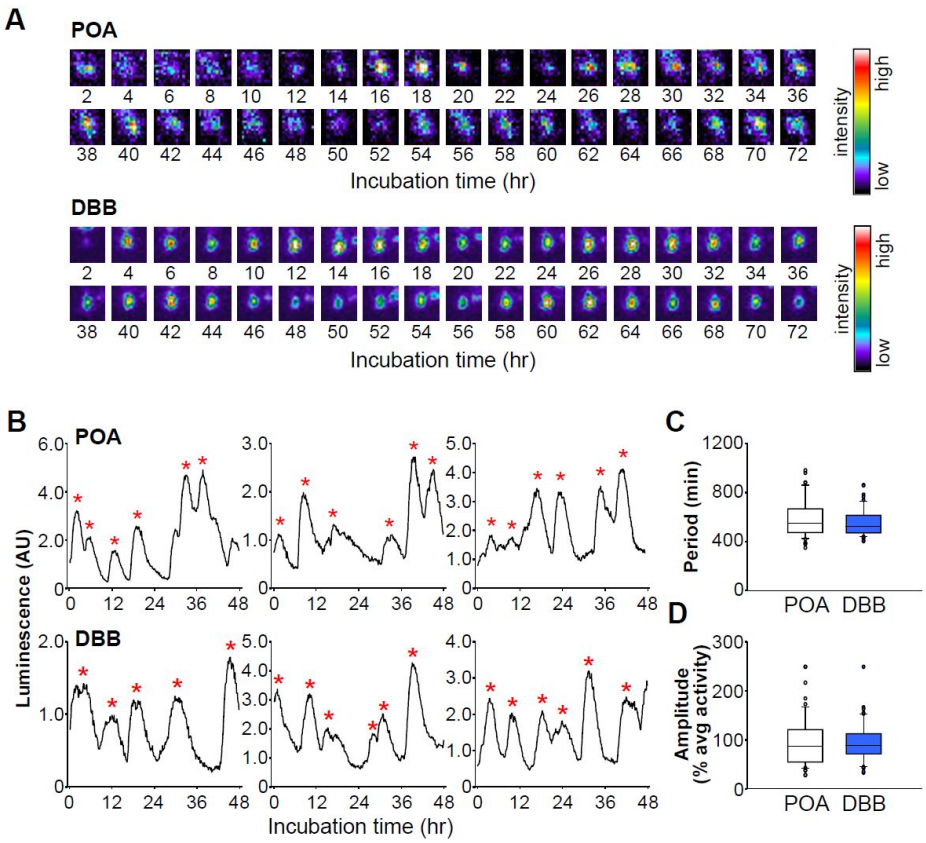

A
POA
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72
Incubation time (hr)
intensity
high
low
DBB
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72
Incubation time (hr)
intensity
high
low
B
POA
DBB
Luminescence (AU)
0 12 24 36 48
Incubation time (hr)
C
Period (min)
0 400 800 1200
POA DBB
D
Amplitude (% avg activity)
0 100 200 300
POA DBB

Figure 10. Ultradian rhythm of GnRH promoter activities in slice preparation plane. (A) Time lapse luminescence images of a single GnRH neuron (upper, coronal; lower, sagittal). (B) Representative bioluminescence profiles of GnRH promoter activity in the cultures of coronally cut (upper panels) and sagittally cut (lower panels) POA slices. (C, D) Peak period (C) and peak amplitude (D) of the GnRH promoter activity observed in GnRH neurons from coronal and sagittal slice cultures were compared (n = 27-46 cells from 2 batches). In the box plots (C, D), data are expressed as median (line), interquartile (box), 10% and 90% percentiles (bar).

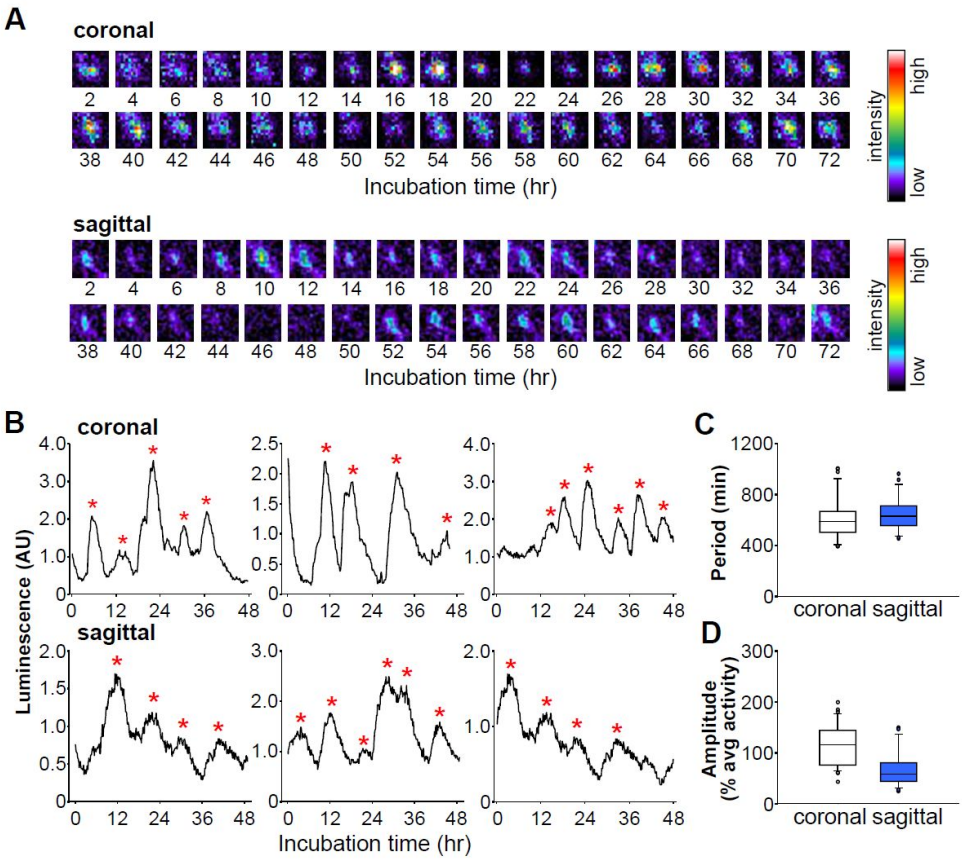

A
coronal
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72
Incubation time (hr)
intensity
high
low
sagittal
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72
Incubation time (hr)
intensity
high
low
B
coronal
sagittal
Luminescence (AU)
Incubation time (hr)
C
Period (min)
coronal sagittal
D
Amplitude (% avg activity)
coronal sagittal

Figure 11. Effects of slice culture and the preparation age on ultradian GnRH promoter activities. (A) Time lapse luminescence images of GnRH neuron. (upper, cultured neonate; middle, acute neonate; lower, acute adult) (B) Representative bioluminescence profiles of GnRH promoter activity monitored in the POA region from cultured neonatal slices (upper panels), acutely prepared neonatal slices (middle panels), and acutely prepared adult slices (lower panels). (C, D) Peak period (C) and peak amplitude (D) of GnRH promoter activity observed in GnRH neurons from cultured neonatal slices (CN), acute neonatal slices (AN), and acute adult slices (AA) were used for comparison (n = 46 cells from 2 batches of CN, 23 cells from 2 batches of AN and 54 cells from 5 batches of AA). In the box plots (B, C), data are expressed as median (line), interquartile (box), 10% and 90% percentiles (bar).

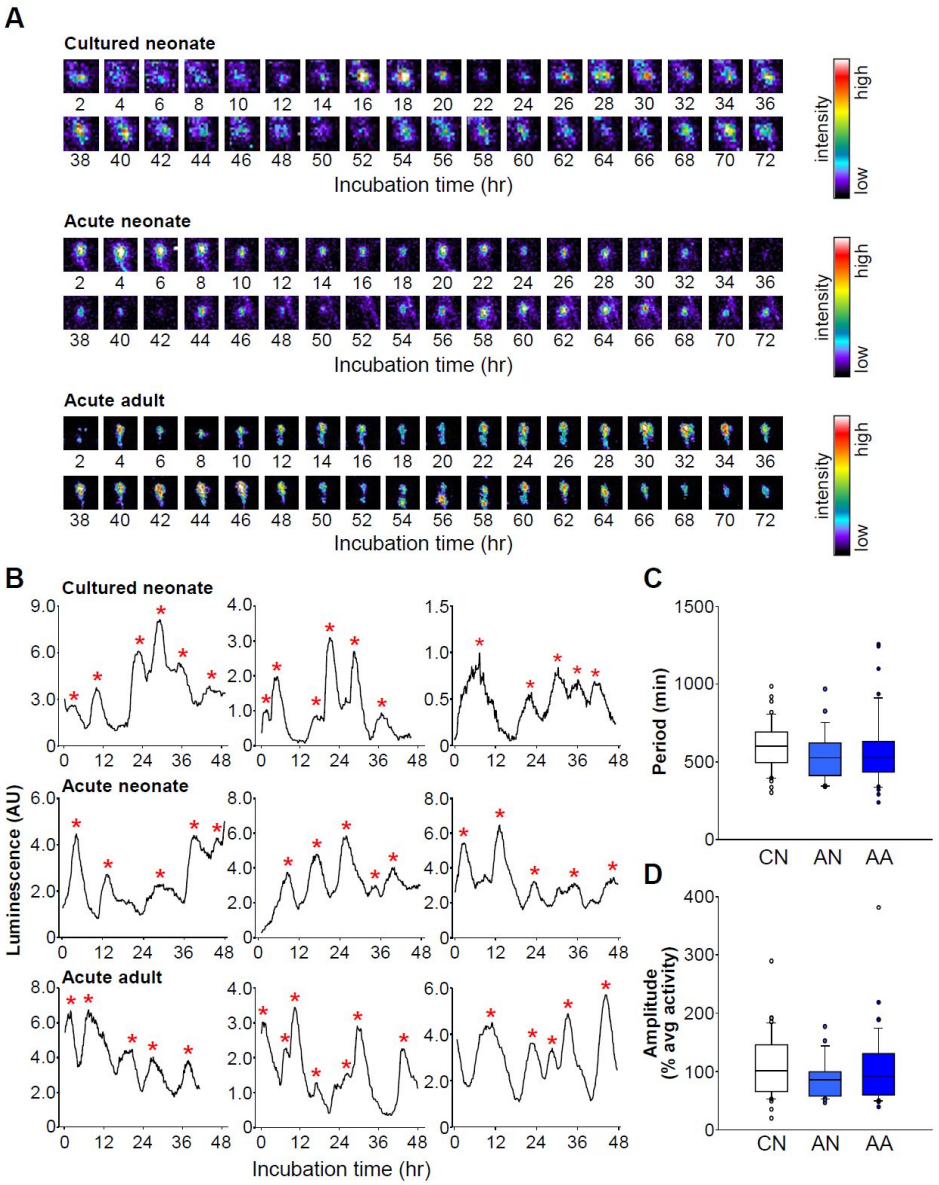

A
Cultured neonate
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72
Incubation time (hr)
intensity
high
low
Acute neonate
Acute adult
B
Cultured neonate
Acute neonate
Acute adult
Luminescence (AU)
Incubation time (hr)
C
Period (min)
CN AN AA
D
Amplitude (% avg activity)
CN AN AA

Figure 12. Effect of TTX and Nimo on the basal oscillations. (A) Representative profiles showing GnRH promoter activities in the presence of vehicle (VEH, upper panels), tetrodotoxin (TTX, middle panels) and nimodipine (Nimo, lower panels). The asterisks indicate peaks. (B, C) Peak period (B) and amplitude (C) in the presence of TTX and Nimo were compared with VEH as a percentage of the pre-treatment average (n = 32–86 cells from 2-3 batches). Data are shown as the mean ± s.e.m.

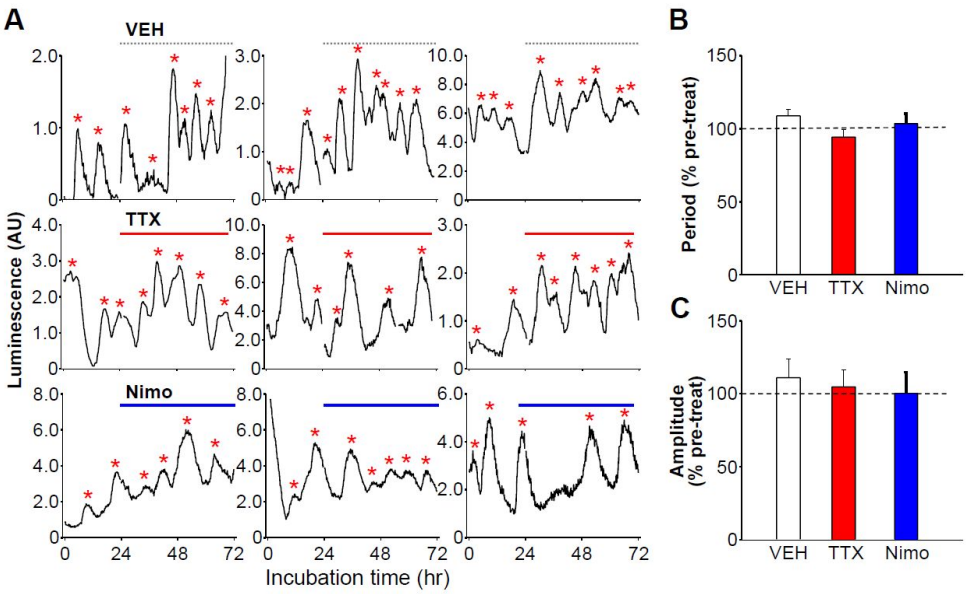

A
VEH
TTX
Nimo
Luminescence (AU)
Incubation time (hr)
B
Period (% pre-treat)
VEH
TTX
Nimo
C
Amplitude (% pre-treat)
VEH
TTX
Nimo

Figure 13. Synchronous GnRH expression and secretion under basal conditions. (A) Synchronization of a GnRH neuronal population. Each row represents an individual neuron (green bar, peak; red bar, nadir). (B) Profile of GnRH secretion. Data are shown as the mean ± s.e.m. Asterisks indicate peaks.

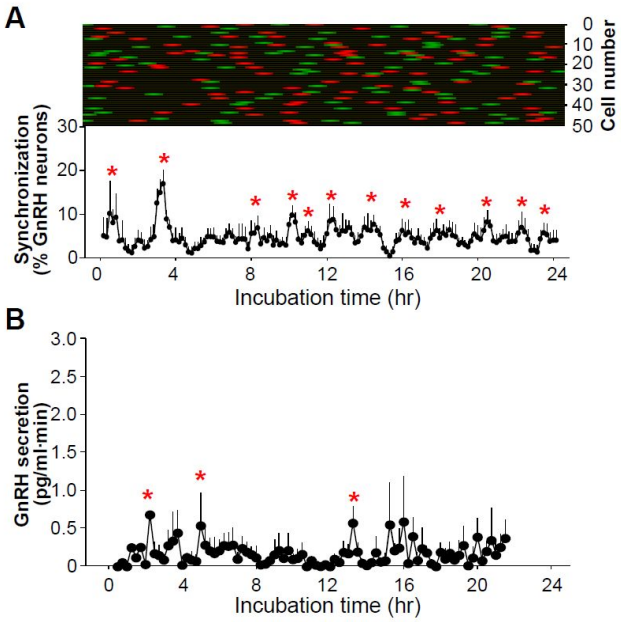

A
0
10
20
30
40
50
Cell number
30
20
10
0
Synchronization
(% GnRH neurons)
0
4
8
12
16
20
24
Incubation time (hr)
B
3.0
2.5
2.0
1.5
1.0
0.5
0
GnRH secretion
(pg/ml·min)
0
4
8
12
16
20
24
Incubation time (hr)

Figure 14. Mode of kisspeptin administration on the synchronization of GnRH gene expression. (A, B) Representative profiles of an individual GnRH neuron applied with indicated stimulation (KISS: kisspeptin, 10 nM for intermittent and 2.5 nM for continuous administration). In intermittent administration, kisspeptin is administered in 15-min on:45-min off. Blue bar, kisspeptin stimulation. Data are shown as raw data (A) or the detrended profile (B). (C) Heatmap plot of normalized detrended luminescence for a representative batch. Each row represents an individual GnRH neuron (green, low; red, high). Synchronization index are shown as the mean ± s.e.m (n = 3-4 batches for each treatment). Gray bars indicate typical range of synchronization reached under basal conditions.

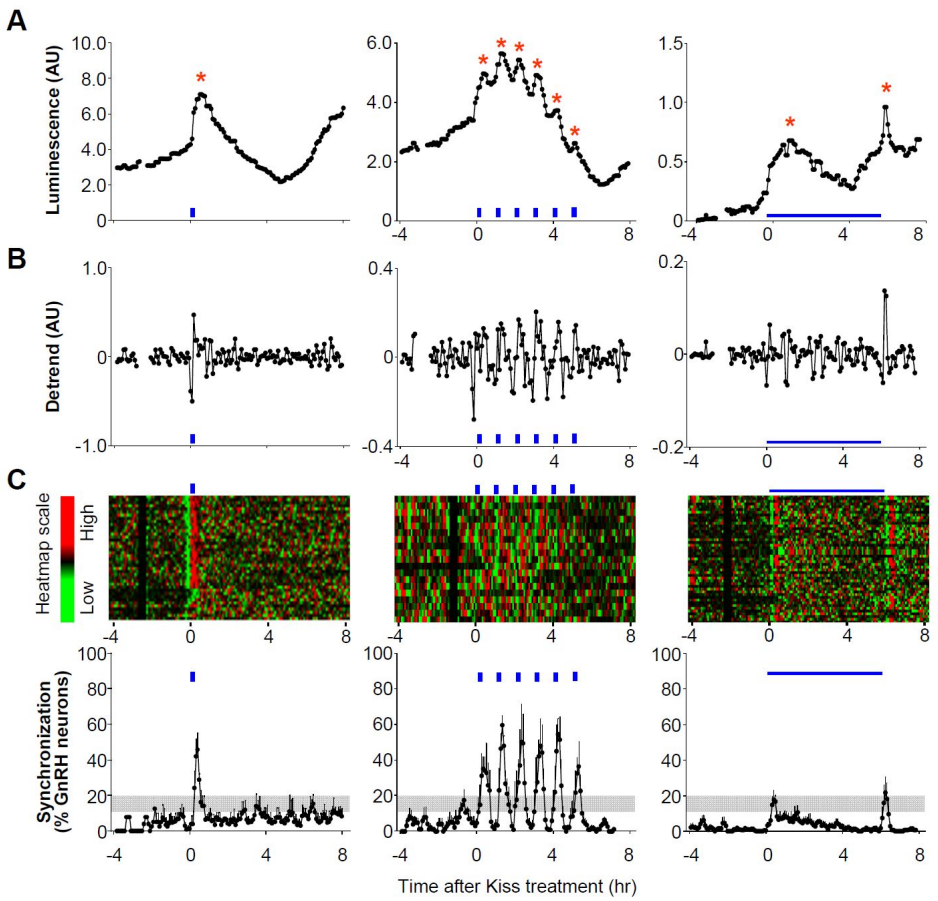

A
Luminescence (AU)
10.0
8.0
6.0
4.0
2.0
0
6.0
4.0
2.0
0
1.5
1.0
0.5
0
-4
0
4
8
B
Detrend (AU)
1.0
0
-1.0
0.4
0
-0.4
0.2
0
-0.2
C
Heatmap scale
High
Low
Synchronization
(% GnRH neurons)
100
80
60
40
20
0
Time after Kiss treatment (hr)

Figure 15. Mode of kisspeptin administration on the pulsatile GnRH secretion. (A-C) GnRH secretions in perifused media are determined at a 15-min interval. Cultured POA slices are applied with single pulse (A), six intermittent (B) or continuous kisspeptin (C) (blue bar, pulse of indicated stimulation).

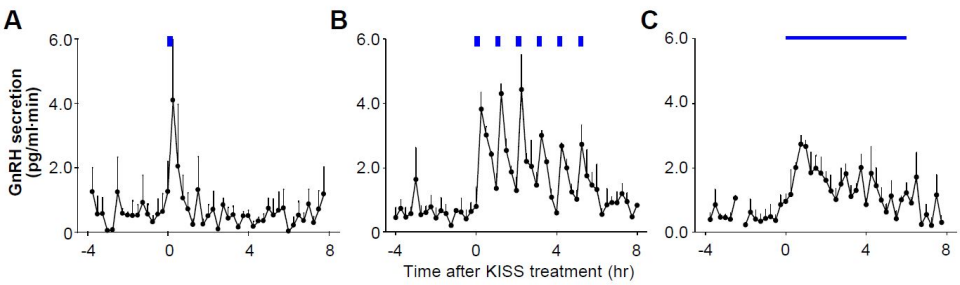

A
B
C
GnRH secretion (pg/ml·min)
6.0
4.0
2.0
0
0.0
-4
0
4
8
Time after KISS treatment (hr)

Figure 16. Specific synchronization of GnRH expression by kisspeptin. (A, B) Representative profiles of an individual GnRH neuron applied with kisspeptin (KISS), senktide (SENK) or dynorphin A (DYN) in a pulsatile manner (15-min on: 45-min off of 10 nM kisspeptin, 50 μM SENK and 50 μM DYN). Data are shown as raw data (A) or the detrended profile (B). (C) Heatmap plot of normalized detrended luminescence for a representative batch. Each row represents an individual GnRH neuron (green, low; red, high). Synchronization index are shown as the mean ± s.e.m (n = 3-4 batches for each treatment). Gray bars indicate typical range of synchronization reached under basal conditions.

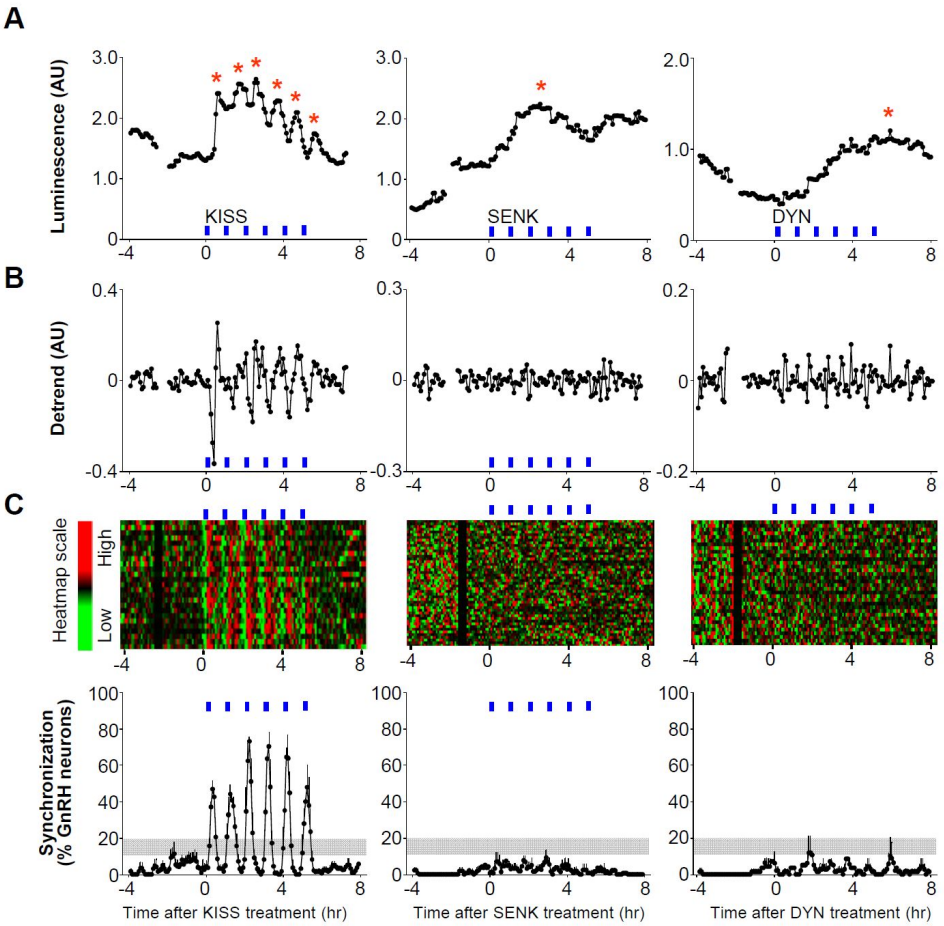

A
B
C
Luminescence (AU)
Detrend (AU)
Heatmap scale
High
Low
KISS
SENK
DYN
Synchronization (% GnRH neurons)
Time after KISS treatment (hr)
Time after SENK treatment (hr)
Time after DYN treatment (hr)

Figure 17. Specific reinforcement of GnRH secretion by kisspeptin. (A-C) GnRH secretions in perifused media are determined at a 15-min interval. The POA slice cultures are administered with kisspeptin (KISS, A), senktide (SENK, B) or dynorphin A (DYN, C) in a pulsatile manner (15-min on: 45-min off of 10 nM kisspeptin, 50 μM SENK and 50 μM DYN).

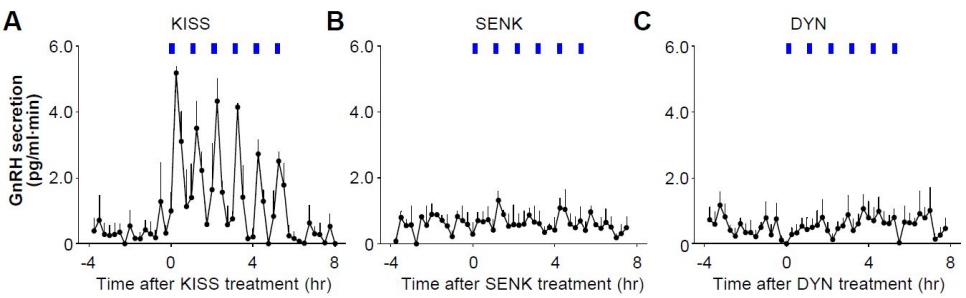

A
KISS
B
SENK
C
DYN
GnRH secretion (pg/ml·min)
6.0
4.0
2.0
0
-4
0
4
8
Time after KISS treatment (hr)
Time after SENK treatment (hr)
Time after DYN treatment (hr)

Figure 18. Synchronization of GnRH gene expression via kisspeptin-GPR54-PKC pathway. (A, B) Representative profiles of an individual GnRH neuron stimulated with intermittent kisspeptin with the vehicle or indicated antagonists (15a, kisspeptin antagonist (30 μM); Gö 6983, a PKC inhibitor (10 μM)) are shown as raw data (A) or the detrended profile (B). (C) Heatmap plot of normalized detrended luminescence for a representative batch. Each row represents an individual GnRH neuron (green, low; red, high). Synchronization index are shown as the mean ± s.e.m (n = 3-4 batches for each treatment). Gray bars indicate typical range of synchronization reached under basal conditions.

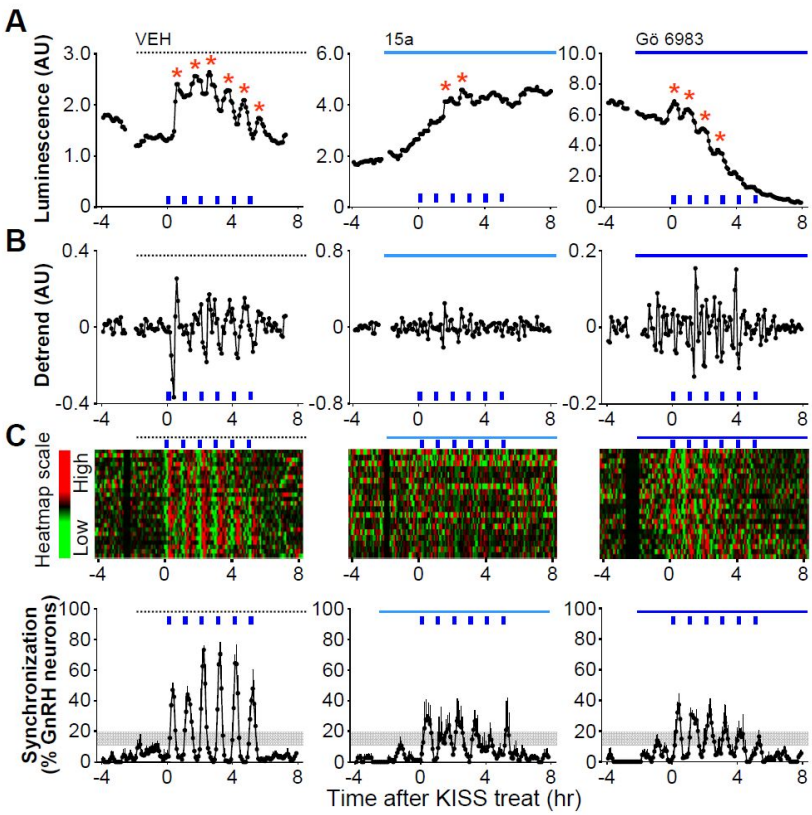

A
VEH
15a
Gö 6983
Luminescence (AU)
B
Detrend (AU)
C
Heatmap scale
High
Low
Synchronization (% GnRH neurons)
Time after KISS treat (hr)

Figure 19. Effects of kisspeptin antagonist and PKC inhibitor on pulsatile GnRH secretion. (A) GnRH secretions in perifused media (blue bar, kisspeptin administration; line, vehicle or indicated drugs) containing the indicated drugs 15a, kisspeptin antagonist (30 μM); Gö 6983, a PKC inhibitor (10 μM). (B, C) Maximum value of synchronization (B) and GnRH secretion (C) (n = 52 – 72 cells from 3-4 batches per treatment). (D) Cumulative GnRH secretion during 6-hr kisspeptin treatment. Data are presented as mean ± s.e.m (n = 3-4 batches for each treatment).

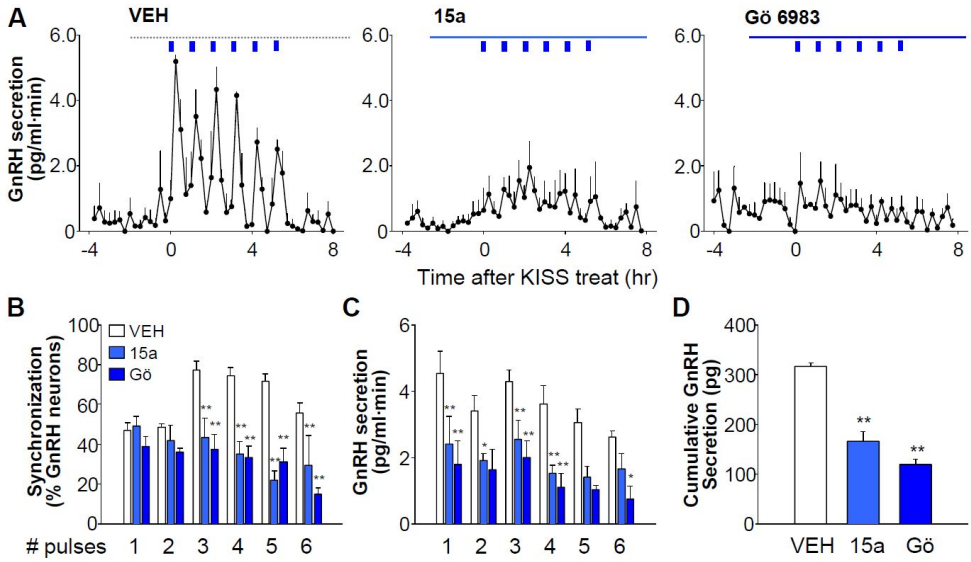

A
VEH
15a
Gö 6983
GnRH secretion (pg/ml·min)
Time after KISS treat (hr)
B
Synchronization (% GnRH neurons)
VEH
15a
Gö
pulses
C
GnRH secretion (pg/ml·min)
D
Cumulative GnRH Secretion (pg)
VEH
15a
Gö

Figure 20. Contribution of *de novo* protein synthesis and exocytosis in synchronous GnRH pulse generation. (A, B) Representative profiles of an individual GnRH neuron with pulsatile kisspeptin stimulation in the presence of the vehicle or indicated drugs (CHX, cycloheximide (100 μM); BFA, Brefeldin A (10 μg/ml)). Data are shown as raw (A) or the detrended profile (B). (C) Synchronization of GnRH neuronal population. Heatmap plot of normalized detrended luminescence of a representative batch is shown above. Each row represents an individual GnRH neuron (green, low; red, high). Data are shown as the mean ± s.e.m (n = 4-5 batches for each treatment).

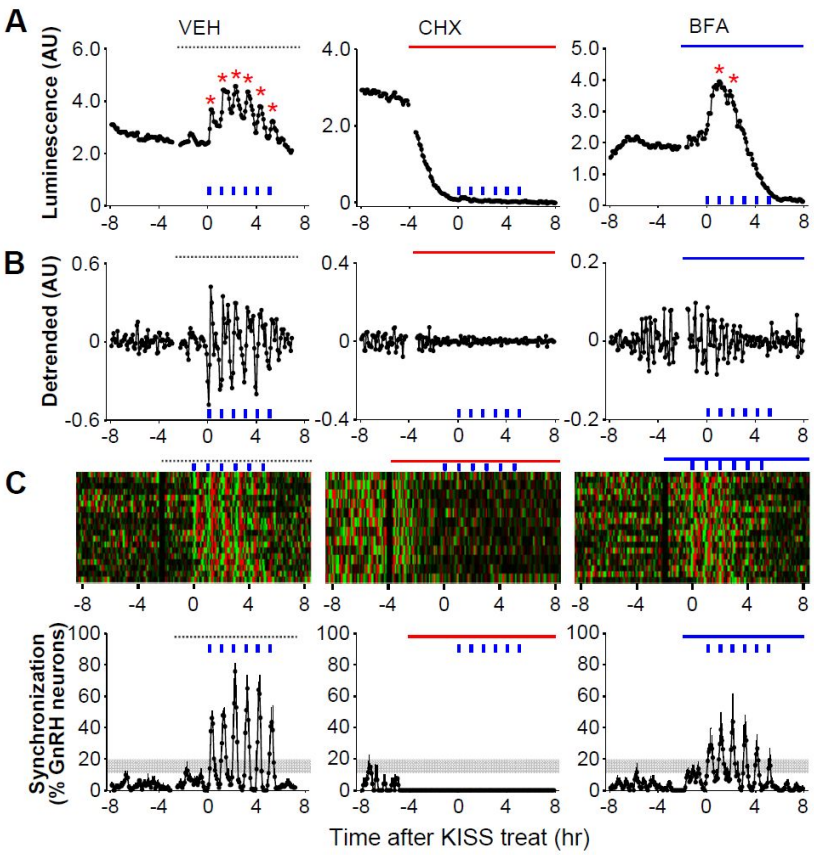

A
VEH
CHX
BFA
Luminescence (AU)
B
Detrended (AU)
C
Synchronization (% GnRH neurons)
Time after KISS treat (hr)

Figure 21. Effects of protein synthesis blocker and exocytic pathway blocker on kiss-synchronized GnRH pulse generation. (A) GnRH secretion in perifused media (blue bar, kisspeptin; CHX, cycloheximide (100 μM); BFA, Brefeldin A (10 μg/ml)). (B, C) Maximum synchronization (B) and GnRH secretion (C) after kisspeptin pulses (n = 65–89 cells from 4-5 batches per treatment). (D) Cumulative GnRH secretion during 6-hr kisspeptin treatment. Data are shown as the mean ± s.e.m (n = 4-5 batches for each treatment).

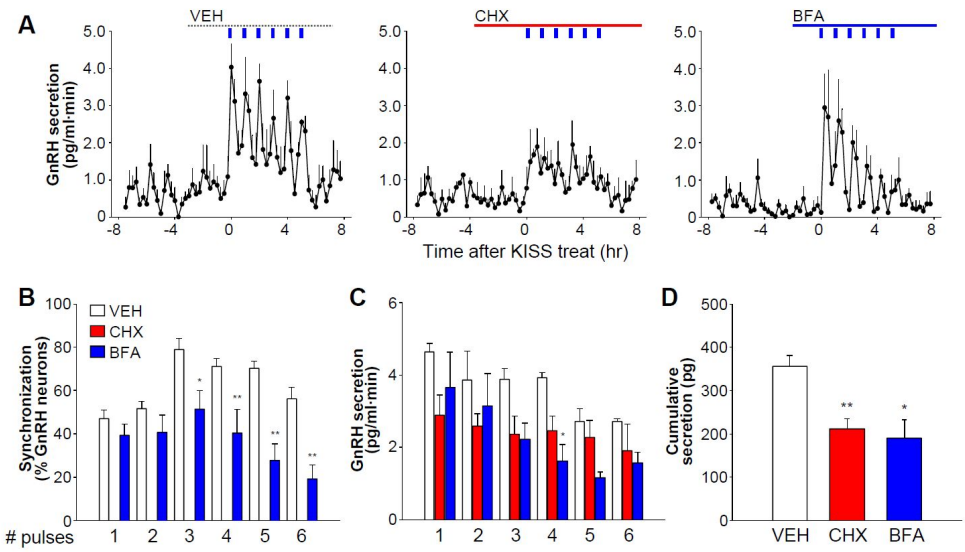
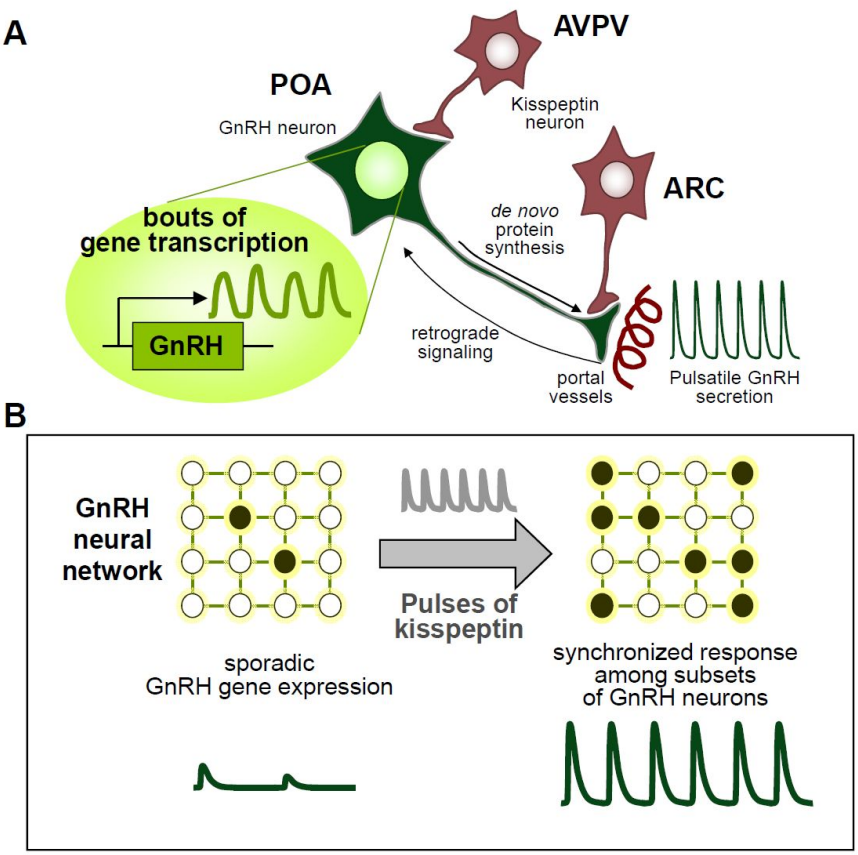

A
VEH
CHX
BFA
GnRH secretion (pg/ml·min)
Time after KISS treat (hr)
B
VEH
CHX
BFA
Synchronization (% GnRH neurons)
pulses
C
GnRH secretion (pg/ml·min)
D
Cumulative secretion (pg)
VEH
CHX
BFA

Figure 22. A hypothetical model for GnRH pulse generator synchronized by rhythmic kisspeptin. (A) GnRH neuron, which secrets GnRH neurohormone into the hypothalamic-pituitary portal vessels, is innervated by kisspeptinergic neurons, of which cell bodies are located in the either anteroventral periventricular nucleus (AVPV) or arcuate nucleus (ARC) of the hypothalamus. (B) Under basal, unstimulated conditions, bouts of GnRH gene expression are sporadic (left panel) and intermittent stimulation of kisspeptin dramatically recruits subset of GnRH neurons to synchronize GnRH gene expression which is then associated with pulsatile GnRH secretion (right panel). Each circle represents a single GnRH neuron in the GnRH neural network. GnRH neurons in sync are presented as closed circles, and out of sync are open circles. *De novo* protein synthesis and communication by GnRH itself or releasable factor may mediate a synchronized response among subsets of GnRH neurons (*A*).


A
AVPV
POA
GnRH neuron
Kisspeptin neuron
ARC
bouts of gene transcription
de novo protein synthesis
GnRH
retrograde signaling
portal vessels
Pulsatile GnRH secretion
B
GnRH neural network
Pulses of kisspeptin
sporadic GnRH gene expression
synchronized response among subsets of GnRH neurons

DISCUSSION

The present study addressed the longstanding issue on the mechanism of GnRH pulse generator, a classic example of ultradian biological rhythm. Under basal, unstimulated conditions, bouts of GnRH promoter activity are sporadic in a single GnRH neuron, which is synchronized among 10-20% of GnRH neurons in an approximately 2-hour interval. Intermittent kisspeptin administration recruits subsets of GnRH neurons to synchronize in association with pulsatile GnRH secretion. These findings clearly demonstrate a critical role of kisspeptin in the GnRH pulse generation.

GnRH gene transcription of a single neuron in the cultured hypothalamic slices exhibited ultradian bouts. The ultradian oscillations in GnRH promoter activity occurred with approximately 10-hr interval, while quite irregular interpulse was observed in each interpulse interval (Fig.7, 8). The oscillations in GnRH promoter activity in GT1 cells, GnRH neuron-derived cell line, have been previously reported by Frawley and colleagues (Nuñez et al., 1998; Nuñez et al., 2000; Vazquez-Martinez et al., 2001). However, the interpulse intervals of GnRH promoter activity monitored in a single GT1 were not consistent in several articles by the same group, ranging from approximately 1 hr (Nuñez et al., 2000; Vazquez-Martinez et al., 2001) to 10 hr (Leclerc and Boockfor, 2007). The unstable interpulse interval observed in the GT1 cells may reflect the differences the culture condition or passage of cell line. In this regards, it can be postulated that the dynamic fluctuation of GnRH promoter activity may originate from the different

source in either GT1 cell line or hypothalamic GnRH neurons. For example, the oscillation observed in GT1 cells were suppressed by the application of nimodipine (Vazquez-Martinez et al., 2001), an L-type calcium channel blocker, while nimodipine application did not affect the ultradian oscillation of GnRH gene transcription in my model (Fig. 12).

Spontaneous oscillations observed in GnRH neuron *in vitro* and GT1 cell lines have suggested that GnRH neuron themselves harbors intrinsic oscillator which may synchronize to yield pulsatile GnRH secretion by a yet unknown mechanism (Moenter et al., 2003). For example, firing of action potential exhibits an uneven temporal dynamics which forms discrete bursts (Moenter et al., 2003; Nunemaker et al., 2003). Intracellular calcium oscillation observed by single-cell calcium imaging also suggests that GnRH neurons derived from embryonic olfactory placode can synchronize in an interpulse interval comparable to that of GnRH secretion *in vivo* without external stimulation (Moore et al., 2002). However, most of these experiments have been conducted without simultaneous measurement of GnRH secretion. Molecular mechanisms driving aforementioned oscillation are still uncharacterized to limit the further assessment of the importance of the GnRH neuron intrinsic oscillations in pulse generation.

In the present study, I simultaneously measured the GnRH promoter activity in a single-cell level and GnRH secretion in the perifused media (Fig. 6). The spontaneous oscillation in single GnRH neuron synchronizes at approximately 2-hr interval, only partly associated with pulsatile GnRH secretion (Fig. 13). The weak pulsatility observed in the cultured POA slices

rather resembles immature pulsatility of prepubertal animal (Sisk and Foster, 2004). It is well established that cell bodies of GnRH neurons are scattered through the POA region and that connection between GnRH neurons are only occasionally found (Witkin et al., 1982; Witkin and Silverman, 1985). In this regards, it is natural to raise a question whether GnRH neuronal population themselves are enough to generate adult-like hourly pulsatility.

The present study suggests that ultradian kisspeptinergic inputs may operate as a critical pacemaker in the orchestration of the bursts of GnRH biosynthesis as well as secretion which constitute GnRH pulse generation. Accumulating evidence has suggested that kisspeptin-GPR54 signaling may play a role in GnRH pulse generation. The inhibition of hypothalamic kisspeptin signaling suppresses pulsatile GnRH/LH secretion (Li et al., 2009; Roseweir et al., 2009). And administration of kisspeptin reset the interpulse interval of LH pulsatility in the human (Chan et al., 2011). On the other hand, the level of kisspeptin itself was shown to be episodic and temporally coincide with or advance the GnRH pulses in the rhesus monkey (Keen et al., 2009). However, it remains largely unexplored how GnRH neurons may respond to kisspeptin stimulation in terms of synchronized pulse generator activity. Here, I have shown that the synchronization was enhanced by kisspeptin stimulation with pulsatile GnRH secretion which responds to each kisspeptin stimulations (Fig. 14, 15). Whereas single pulse or continuous administration of kisspeptin was insufficient to evoke pulsatile synchronization and secretion of GnRH, indicating that pulsatile kisspeptin stimulation is required to generate

pulsatility of GnRH neuronal population.

The intrinsic property of GnRH neurons appears to be important in this process. Spontaneous and cell-autonomous ultradian rhythmicity in GnRH gene transcription and stochastic synchronization among subset of GnRH neurons also appear to be targets of kisspeptin, and this is further supported by their heterogeneous responsiveness to kisspeptin. In response to the initial kisspeptin pulse, transient induction of promoter activity was observed in a subset (approximantely 40%) of GnRH neurons (Fig. 14C middle). Further rounds of kisspeptin stimulation recruited subsets of GnRH neurons to participate in the synchronization process. The source of this heterogeneity may result from variable GPR54 expressions in GnRH neuronal population. Although co-immunostaing have been yet possible due to lack of suitable antibody against GPR54, immunohistochemical analysis of GnRH neurons in GPR54 KO mice which is designed to express LacZ instead of GPR54 revealed that 30-70% of GnRH neuron were simultaneously stained with Xgal (Herbison et al., 2010). This heterogeneity is also reflected in an electrophysiological responsiveness to kisspeptin stimulation, where 30-40% GnRH neurons derived from prepubertal animal respond to kisspeptin (Han et al., 2005). Approximately half of GnRH neurons in mediobasal hypothalamus respond to kisspeptin while other subsets of GnRH neurons respond to DHPG, a group I metabotropic glutamate receptor agonist (Dumalska et al., 2008). On the other hands, it is a little controversial whether molecular nature of GPR54 expression in GnRH neuron and kisspeptin responsibility in adult

GnRH neuron (Han et al., 2005). Interaction between subsets of GnRH neurons for recruitment as well as their contributions in GnRH pulse generation should be further investigated.

Kisspeptin neurons in the ARC are tripeptidergic neurons, which co-expresses NKB and DYN. Kisspeptin can upregulate LH secretion, whereas DYN plays an opposite role (Navarro, 2012). The effect of NKB or its analog senktide usually activates LH secretion via kisspeptinergic signaling, while the exact effect depends on occasions. Interestingly, kisspeptin neurons in the ARC expresses receptor for NKB and DYN (called NK3R and KOR, respectively) (Navarro et al., 2009). Therefore, a feedback loop comprised of positive regulator NKB and negative regulator DYN in the kisspeptin producing neurons of the ARC has been proposed (Navarro et al., 2009; Lehman et al., 2010), while the mechanism through might mediate these effects is unknown. Nonetheless, the effect of these neuropeptides on GnRH neurons has not been thoroughly examined. In contrast to kisspeptin, pulses of NKB and DYN did not elicit any detectable changes in the GnRH neurons in the cultured POA slices (Fig. 16, 17), implying that *in vivo* effect of NKB and DYN may originate from its influence on kisspeptin neurons in the ARC. Together these results suggest that pulsatile kisspeptin is a direct pacemaker on GnRH neuronal population, whereas NKB and DYN may play a role in the feedback loop of Kiss/NKB/DYN neurons.

This effect of kisspeptin may also be important for the key role of kisspeptin-GPR54 signaling during the onset of puberty (Roa et al., 2008;

Oakley et al., 2009; Navarro and Tena-Sempere, 2011). Prepubertal vertebrates exhibit a quiescent period of GnRH secretion, and after puberty, pulsatile GnRH secretion increases and remains active (Sisk and Foster, 2004; Herbison, 2006). GPR54 is activated during the sexual maturation process, playing a key role in pubertal onset and reproductive functioning in many species (Roa et al., 2008; Oakley et al., 2009). The expression of kisspeptin and GPR54, and particularly the kisspeptin immunoreactivity juxtaposed to the GnRH fibers, are dramatically induced with pubertal onset, just at time of pubertal activation of GnRH pulse generation (Clarkson et al., 2010). It would certainly appear that increased kisspeptin output – which may itself be episodic (Keen et al., 2008; Navarro et al., 2009; Lehman et al., 2010) – could play an important role in the timely activation of the GnRH pulse generator. In this regards, it is notable that kisspeptin is able to evoke dramatic synchronization among subset of GnRH neurons derived from the sexually immature mouse (Fig. 14), which is strongly associated with pulsatile secretion of GnRH. Therefore GnRH neurons in organotypic culture appear to already posses the machinery for GnRH pulse generation even before pubertal maturation. It would be an important future question whether the generation of kisspeptin pulses is initiated during the onset of puberty.

In conclusion, I show that rhythmic kisspeptin stimulation of GnRH neurons evokes pulsatile GnRH release, and that the communication among hypothalamic neural network then mediates a synchronized response among subsets of GnRH neurons (Fig. 22). The capability of

kisspeptin to synchronize GnRH pulse generation in neonatal cultures of GnRH neurons highlights the importance of pulsatile kisspeptin input as an ultimate pacemaker for the generation of ultradian GnRH pulses. Using GnRH pulse generation as a model system, these findings provide valuable insight into biology of the ultradian rhythms widely found in neuroendocrine systems.

CHAPTER 2

Role of Bmal1, a core clock component, in the generation of synchronous GnRH pulses

ABSTRACT

Diverse biological rhythms are found almost at every level of the biological system. However, hierarchical interaction between different periods of biological rhythms remains unknown. To explore the interaction between circadian and ultradian rhythms, I examined the ultradian GnRH gene transcription and kisspeptin-synchronized GnRH pulse generator in cultured preoptic area (POA) slice derived from GnRHp-dsLuc transgenic (TG) mice lacking *Bmal1*, a core transcriptional factor of molecular circadian clock. Spontaneous ultradian rhythm of GnRH promoter activity in an individual GnRH neuron and stochastic synchronization among the subset of GnRH neurons under basal conditions were rarely altered in *Bmal1* knockout (KO) mice. Interestingly, POA slice derived from mouse lacking Bmal1 exhibited impaired pulsatility in GnRH secretion during intermittent kisspeptin stimulation. Response of *Bmal1* KO slice to continuous application of kisspeptin was comparable the with the wildtype slice, implying that proper function of *Bmal1* may have implications in the processing, storage, or secretion of the GnRH peptide and that impaired pulsatility was not resulted from simply being incapable of kisspeptin response. In conclusion, the cellular circadian clockwork may organize the maintenance of GnRH pulse generator, thereby providing a novel insight on the hierarchical interaction between biological rhythms.

Keywords: Circadian rhythm, ultradian rhythm, hierarchical interaction, GnRH pulse generator, Bmal1

INTRODUCTION

Biological rhythms oscillate with a several fold of periods, consisting of hierarchy of biological rhythm. While majority of biological rhythm remain unclear on their driving force, circadian rhythm, with approximately 24-hr period, is exceptionally well dissected for its molecular nature. Molecular circadian clock in mammals are based on interlocked transcription-translational loop (Fig. 2; Takahashi et al, 2008; Bellet and Sassone-Corsi, 2010; Son et al., 2011). Activating dimer consisted of CLOCK and BMAL1, binds to E-box elements in the promoter regions of clock-controlled genes (CCGs) and activated transcription of the CCGs. *Per1, 2* and *Cry1,2*, negative regulators in the loop, are transcriptionally activated by CLOCK:BMAL1 heterodimer via E-box elements within their promoter. *Per*s and *Cry*s subsequently repress CLOCK:BMAL1 activated transcription and complete a cycle of molecular feedback loop. Additional feedback loop composed of *Ror*s and *Rev-erb*s also contributes to normal functioning of circadian clockwork.

Indeed, previous studies have suggested that there can be functional crosstalks between circadian and ultradian rhythms. For example, in the HPA axis activity, the circadian rhythms found in hormonal profiles are well known to be interlocked with their ultradian oscillations (Lightman and Conway-Campbell, 2010). Circadian or ultradian feeding regime affects the expression of circadian gene in the liver of hamster with ultradian activity (van der Veen et al., 2006). In this regards, it is noteworthy that

reproductive system regulated by hypothalamus-pituitary-gonad (HPG) axis has long been associated with circadian rhythm. More than 60 years ago, Everett and Sawyer revealed that barbiturate application on the afternoon of diestrous delayed the LH surge by one day (Everett and Sawyer 1950), suggesting a circadian component on the controlling HPG axis. Recent progress in the molecular genetics of circadian clock suggests that molecular circadian clockwork appears to be crucial for normal functioning of the reproductive axis; *Clock* mutant mice are subfertile and exhibit abnormal estrous cycles as a result of defects at the hypothalamic level (Miller et al., 2004). Genetic disruption of *Bmal1*, a heterodimeric partner of Clock, also lead to delayed onset of puberty, disrupted estrus cyclicity, and reduced gonad weight (Boden et al., 2010).

GnRH neuron, a central integrator for HPG axis, contains and are under control of molecular circadian clockwork. Circadian expression of molecular circadian clock in the GT1-7 cell, immortalized GnRH neurons, have claimed from in vitro studies (Gillespie et al., 2003; Chappell et al., 2003). More importantly it has been argued that cellular circadian clock machinery may be required for spontaneous GnRH pulsatility in its release in GT1 cell line (Chappell et al., 2003). Additionally, circadian clock is implied in the regulation of kisspeptin responsibility (Zhao and Kriegsfeld, 2009; Tonsfeldt et al., 2011).

Therefore, I designed the present study to elucidate a possible role of molecular clockwork in spontaneous and kisspeptin-evoked GnRH pulse generation. In order to appreciate the contribution of circadian clockwork, I

prepared cultured hypothalamic slices by use of animals lacking *Bmal1*, a core transcription activator of molecular circadian clock (Bunger et al., 2000). Cell-autonomous clockwork is largely impaired in the animal model, providing valuable system to assess contribution of molecular clockwork in the generation of GnRH pulsatility.

MATERIALS AND METHODS

Animal care and handling. C57BL/6J mice were housed in temperature-controlled (22~23°C) quarters under a 12-hr light and 12-hr dark (LD) photoperiod (light-on at 8:00 a.m.); standard mouse chow and water were available *ad libitum*. *Bmal1* knockout mouse (Bunger et al., 2000) was generously provided by Dr. Marina Antoch (Roswell Park Cancer Institute) and Dr. Karyn Esser (University of Kentucky). All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Slice culture. The materials for slice culture were obtained from Invitrogen (Carlsbad, CA). The slice culture was prepared as previously reported with minor modifications (Yamaguchi et al., 2003). Briefly, the neonate genotype was determined on postnatal day 3-5 by PCR from tail genomic DNA. Transgenic mice (postnatal day 5-7) were anesthetized with ether, and the removed brains were immediately transferred to ice-cold Gey's balanced salt solution (GBSS; with 10 mM HEPES, 30 mM glucose) bubbled with 5% CO_2 and 95% O_2. The coronal or sagittal brain slices (400-μm thick) were made with a vibratome (Campden Instruments, Leicester, UK) and the brain slices were carefully dissected under a stereomicroscope to minimize the outside regions of the preoptic area (POA), which located at 3.10 to 3.50 mm according to the Paxinos coordinate description (postnatal day 6) (Paxinos et al., 2007). The POA region was clearly distinguished from the

suprachiasmatic nucleus, located at 4.10 to 4.60 mm, and the arcuate nucleus, 4.80 to 6.00 mm. For the adult slices, brain slices containing the POA were sectioned at 200 μm using a vibratome. The POA explants (approximately 1 mm long and 1 mm wide) were maintained on a membrane (Millicell-CM, Millipore) dipped into a culture medium (50% minimum essential medium, 25% Hank's balanced salt solution, 25% horse serum, 36 mM glucose, and 100 U/ml penicillin-streptomycin) at 36°C. For the experiments which included the measurement of GnRH secretion, POA slices from four neonatal mice were maintained on a single membrane. The medium was changed every three days by lifting the Millicell culture membrane and placing it into a new culture dish containing fresh medium.

Single-cell real-time bioluminescence monitoring. The bioluminescence from the POA cultures was monitored as previously reported with slight modifications (Yamaguchi et al., 2003). The bioluminescence from the POA cultures was monitored with a Cellgraph (AB-1000, ATTO, Tokyo, Japan), a specialized microscope for bioluminescence observation, equipped with a Nikon S Fluor 10X objective lens (NA, 0.75). The POA slice cultures were maintained in a customized chamber (Live Cell Instruments, Seoul, Korea) and kept in an incubating unit inside the Cellgraph (36°C, 5% CO_2 balanced with air and humidified). One millimolar D-luciferin (Promega) was included in the recording medium (DMEM:F12 supplemented with 1X N2 supplement, 36 mM glucose, 100 U/ml penicillin-streptomycin). Images of 4 min exposure duration were acquired at 5 min intervals using the Cellgraph

Controller (ATTO). The image acquisition conditions were as follows: Cooling temperature: -80°C; Binning: 1X1; Readout rate: 1 MHz at 16-bit; Output amplifier: electron multiplying; Electron multiplier gain: 200; and Pre-amplifier gain: 1.0. The acquired bioluminescence images were analyzed using MetaVue (Molecular Devices, Sunnyvale, CA). All of the analyses were restricted to cells that were clearly discriminable from adjacent cells. Luminescence intensity was measured within a region of interest defined manually for each cell and filtered through a median filter to eliminate cosmic-ray-induced noise. Average value of the region of interest was presented as arbitrary unit (A.U.) after background correction. The detrended value was obtained by subtracting the 25-min average from the background-eliminated luminescence intensity.

Intermittent kisspeptin stimulation and measurement of the hormone level. For the simultaneous determination of GnRH secretion with bioluminescence monitoring, the POA cultures were maintained in a customized chamber with two input ports and one output port made of stainless steel. Two NE-1000 syringe pumps controlled by a Syringe Pump Pro program (New Era Pump Systems, Farmingdale, NY) were connected with each of the input ports (void volume, approximately 200 μl). The media in the chamber was withdrawn using a Minipuls Evolution peristaltic pump (Gilson, Middleton, WI) connected with the output port (void volume, approximately 300 μl). For intermittent kisspeptin administration, 6 consecutive kisspeptin pulses (15-min kisspeptin (10 nM) followed by 45-

min media washout) were administered to the slice culture. The recording media was perfused at a flow rate of 2.4 ml/hr and collected every 15 min. The collected media was centrifuged at 12,000 rpm for 3 min and stored at -80°C until radioimmunoassay (RIA). GnRH RIA was performed using Chen–Ramirez GnRH antiserum, CRR13B73 (generously provided by V. D. Ramirez, University of Illinois, IL). The sensitivity at 90% binding was approximately 5 pg/tube. The intra- and inter-assay coefficients of variation were 4–5 and 5–10% for a 10 pg dose of synthetic GnRH, respectively.

Single-cell real-time monitoring data analysis. The pulse peak and nadir of the background-subtracted bioluminescence profile were identified using the Cluster-8 program (Veldhuis and Johnson, 1986). Cluster sizes for peaks and nadir were defined as 3, and the *t* statistics to identify a significant increase or decrease was 2.0. The pulse period of an individual cell was defined as the duration between the first and last peak divided by the number of interpulse intervals. The pulse amplitude was normalized by the average bioluminescence activity. The synchronization of a GnRH neuronal population was calculated as previously described (Moore et al., 2002) with minor modifications. Briefly, the time points when the peaks were detected in each cell were compiled into a single file for the entire neuronal population within the imaged field. The EXCEL program assigned either a 0 (no significant peak) or 1 (significant peak detected) for a given point. This series of 0s and 1s was summed and normalized by the number of cells detected in the culture.

Heatmap plot. For the heatmap plots, detrended bioluminescence intensity data were normalized by standard deviation and then color coded for a higher than 1.5 standard deviation in red, and a lower than 1.5 standard deviation in green. Plots were constructed using HCE3.5 (Seo et al., 2006).

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Statistical significance was assessed by unpaired two-tailed Student's *t*-test or two-way ANOVA. Bonferroni post-test was used for *post hoc* comparison of ANOVA. Statistical significance was set as follows: *, $P<0.05$; **, $P<0.01$.

RESULTS

Knockout of Bmal1, a core transcription factor of circadian clock, on GnRH neuron

Considering the hierarchy between circadian and ultradian rhythms, three plausible modes of interaction between them can be postulated. The first is a circadian-drive model, where machinery of circadian rhythm dictates the oscillation of subordinate ultradian rhythm (Fig. 23A). The second is an ultradian rhythm-driven model, where the oscillation of ultradian rhythm underlies generation of circadian rhythm (Fig. 23B). The last is independent oscillator model, where the circadian rhythm and ultradian rhythm have their own machinery for rhythm generation which operates regardless of each other (Fig. 23C).

In the next set of experiment, I examined whether a defective circadian clock exerts an effect on GnRH pulse generation to elucidate a possible link between the circadian oscillator and ultradian rhythm. I monitored the GnRH promoter activities and the secretory profiles in the POA slice cultures obtained from GnRHp-dsLuc transgenic animals lacking functional BMAL1 (GnRHp-dsLuc X *Bmal1*$^{-/-}$), a key transcriptional regulator of the circadian molecular clock.

First, I determined the status of GnRH neuron under basal conditions. To evaluate the GnRH neuron with genetic ablation of Bmal1, I monitored the bioluminescence from cultured preoptic area (POA) slice derived from

GnRHp-dsLuc transgenic mice with either wild-type or *Bmal1*-KO mouse. GnRH neurons in slice culture from both WT and *Bmal1* KO exhibited a similarly scattered pattern of localization, which is a typical characteristics of GnRH neurons in the hypothalamus (Fig. 24A). *Bmal1*-deficient POA slice culture also contains as many GnRH neurons as WT cultures (Fig. 24B). Usually 20-30 cells were simultaneously monitored in a slice in both genotypes. Average intensity of GnRH promoter-driven bioluminescence was also indistinguishable in both genotypes (Fig. 24C). Therefore, genetic ablation of *Bmal1* does not appear to lead to developmental abnormality, abnormal *ex vivo* maturation or basal requirements for GnRH gene transcription. Additionally, determination of tissue GnRH contents by using radioimmunoassay (RIA) indicates that *Bmal1*-KO POA slice cultures contains as much GnRH as WT POA slice cultures (Fig. 24D), implying normal GnRH peptide formation in *Bmal1*-deficient GnRH neurons.

Next, total RNA was purified from POA slice cultures and subject to real-time RT-PCR analysis. In *Bmal1*-lacking cultures, expression of clock genes, for example, *Rev-erb*α, *Per1* and *Per2* decreased compared with WT cultures (Fig. 25 upper and middle panels). However, mRNA levels of GnRH were indistinguishable between *Bmal1*-KO and WT POA cultures (Fig. 25 lower left panel). mRNA abundance of *GPR54* was slightly increased in *Bmal1*-KO cultures, but was not significant by Student's t-test (Fig. 25 lower right panel) (P=0.166, n=4-6 for each genotype).

Basal ultradian rhythm of GnRH transcription and its synchronization in Bmal1-deficient GnRH neuronal population

To gain insight into the engagement of Bmal1 in the ultradian oscillation of GnRH transcription, I monitored the promoter activity of GnRH by using real-time bioluminescence imaging system. Under basal conditions, the ultradian oscillations in the GnRH promoter activity in the *Bmal1*$^{-/-}$ slices were essentially the same as those seen in the wild-type controls in terms of period and amplitude (Fig. 26A lower panel). The average pulse periods were approximately 10 hr in both genotypes (Fig. 26B). The amplitude of pulses in GnRH gene transcription was also rarely affect by *Bmal1*-ablation (Fig. 26C). Together, these results suggest that that intrinsic ultradian GnRH gene expression is driven by an unidentified oscillator distinct from the circadian clockwork.

Next, I aimed to analyse the synchronization of GnRH neuronal population in cultured POA slices derived from either wildtype or *Bmal1*-KO neonatal mice. The episodes of the GnRH promoter activity monitored in the *Bmal1*-KO POA slice cultures exhibited a stochastically synchronized profile similarly with WT POA slice cultures (Fig. 27A). In both genotypes, the GnRH promoter activities among GnRH neuronal population synchronize 10 times a day, yielding approximately two-hr interval synchronization peaks. Peak level of synchronization were 10-15 % of total GnRH neurons in a cultured POA slice, which lies within a similar range as compared with WT POA slice cultures.

The basal GnRH secretion profiles were determined in the perifused media. In agree with transcriptional synchronization, there was not any noticeable change between WT and *Bmal1* KO POA slice cultures (Fig.27B). GnRH secretion under basal conditions was weakly pulsatile, reaching 3 statistically significant peaks a day in WT and 5 peaks in *Bmal1* KO. The peak level of GnRH pulses were usually 0.5 – 1.0 pg/ml in both genotypes. Together these data suggests that population of GnRH neurons of *Bmal1* KO POA slice cultures maintain a functional machinery for basal activity of transcriptional dynamics and its synchronization as well as secretory pulses, probably because they are not quite actively pulsatile.

Kisspeptin-induced generation of GnRH pulsatility

In the next set of experiments, I aimed to elucidate kisspeptin-induced GnRH pulsatility in cultured POA slice derived from *Bmal1* knockout mice. Six consecutive kisspeptin pulses (10 nM) were applied to cultured POA slice derived from wildtype or *Bmal1* knockout mice. *Bmal1*-deficient cultures exhibited impaired responses to pulsatile kisspeptin administration; they had slightly fewer synchronous bursts of GnRH gene transcription (Fig. 28). Pulses of kisspeptin elicited transient increases in GnRH promoter activity with peaks occurring approximately 20-30 min after the initiation of kisspeptin pulse, while amplitude of response was quite decreased compared with those in WT POA slice cultures (Fig. 28A, B). Relatively weak response in *Bmal1* KO slices lead to slightly decreased

synchronization among GnRH neuronal population (Fig. 28C). Two-way ANOVA analysis revealed that there is significant difference between genotypes, while there were not any significant difference in the each peak synchronization corresponding to each kisspeptin pulses between both genotypes (Fig. 29B, $F_{(2,36)} = 49.70$, $P < 0.0001$ for treatment groups; $F_{(5,36)} = 4.644$, $P = 0.0023$ for number of pulses; $F_{(5,36)} = 2.395$, $P = 0.0566$ for treatment-number of pulses interaction).

Interestingly, significant attenuation in pulsatile secretion was observed in the level of GnRH in the perifused media from *Bmal1* KO POA slice cultures compared with wildtype POA slice cultures (Fig. 29A). The first kisspeptin pulse reinforced distinct pulsatile peak approximately 30 min after the initiation of kisspeptin administration in both genotypes. However, the peak levels of GnRH secretion responding to the intermittent kisspeptin pulses (3^{rd} to 6^{th} kisspeptin pulses) were decreased in *Bmal1* KO slices compared with WT POA slices (Fig. 29A and C, $F_{(2,59)} = 12.29$, $P < 0.0001$ for treatment groups; $F_{(5,59)} = 4.754$, $P = 0.0010$ for number of pulses; $F_{(10,59)} = 0.7231$, $P = 0.6996$ for treatment-number of pulses interaction). Cumulative GnRH secretion also decreased in Bmal1-deficient POA cultures (Fig. 29D). In addition to pulsatility, the cumulative secretion of the GnRH peptide was also less in the *Bmal1*$^{-/-}$ cultures, showing an importance of cellular circadian clock in GnRH pulse generation.

Unaffected responsibility to continuous kisspeptin administration

Next, I aimed to investigate the effect of kisspeptin administration scheme. Continuous kisspeptin (2.5 nM) was applied to the POA slice cultures derived from either *Bmal1* KO or WT neonatal mice. Gradual increase followed by gradual attenuation in GnRH promoter activities were observed in GnRH neurons derived from both genotypes (Fig. 30A, B). Weak synchronization at the initiation and termination of kisspeptin stimulation, in which approximately 20% of total GnRH neurons participate, was also essentially same in *Bmal1* KO slices (Fig. 30C). In addition, the *Bmal1*-deficient POA slice cultures also secreted comparable level of GnRH in response to kisspeptin stimulation compared with WT slices (Fig. 30D). Therefore *Bmal1* KO POA slices appear to be less capable in the maintenance of pulsatility during the repeated kisspeptin stimulation session. These results suggest that the cellular circadian clockwork has implications in the processing, storage or secretion of the GnRH peptide as well as synchronization of GnRH neuronal population, rather than directly induces the ultradian oscillations of GnRH gene transcription

Figure 23. Hypothetical interaction between hierarchical circadian and ultradian rhythms. (A) Circadian-driven model. Circadian oscillation would drive the ultradian oscillation. (B) Ultradian-driven model. Machinery responsible for driving ultradian rhythm underlies the generation of circadian rhythm. (C) Independent oscillator model. Molecular oscillator generating either circadian or ultradian rhythm is independent and does not influence each other.

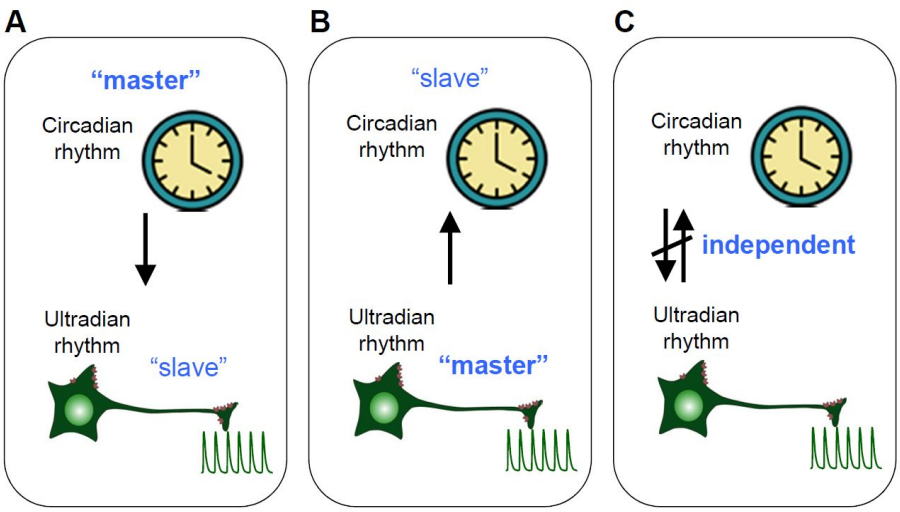

A
"master"
Circadian rhythm
Ultradian rhythm
"slave"
B
"slave"
Circadian rhythm
Ultradian rhythm
"master"
C
Circadian rhythm
independent
Ultradian rhythm

Figure 24. GnRH neurons in the POA slice culture derived from Bmal1 knockout mouse. (A) Bioluminescence image of POA cultures derived from either *Bmal1*$^{+/+}$ (WT) X GnRHp-dsLuc or *Bmal1*$^{-/-}$(KO) X GnRHp-dsLuc crossbred mice. Intensity was shown as pseudocolor. (B) Average number of GnRH neurons per cultured POA slice. (C) Average luminescence of GnRH neurons. (D) GnRH contents of cultured POA slice under basal conditions. Contents of GnRH were normalized by amount of soluble protein. Data are shown as the mean ± s.e.m.

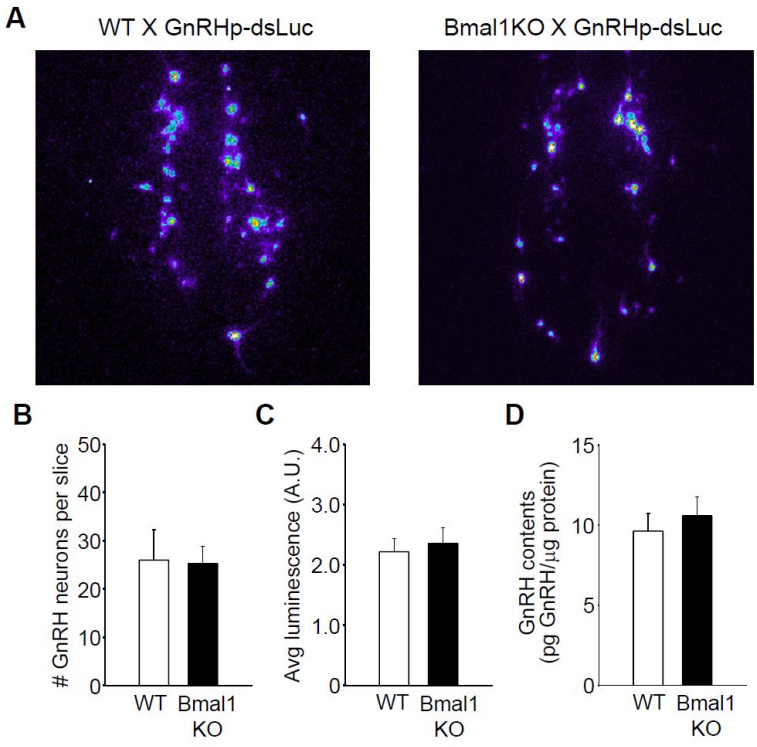

A
WT X GnRHp-dsLuc
Bmal1KO X GnRHp-dsLuc
B
GnRH neurons per slice
50
40
30
20
10
0
WT
Bmal1 KO
C
Avg luminescence (A.U.)
4.0
3.0
2.0
1.0
0
WT
Bmal1 KO
D
GnRH contents (pg GnRH/μg protein)
15
10
5
0
WT
Bmal1 KO

Figure 25. Expression of mRNA in the POA slice culture. Relative mRNA abundance was normalized to average value of WT slice after normalization with internal control, GAPDH. Data are shown as the mean ± s.e.m. (n = 4-6 for each genotypes. *: $P<0.05$ and **: $P<0.01$ by t-test).

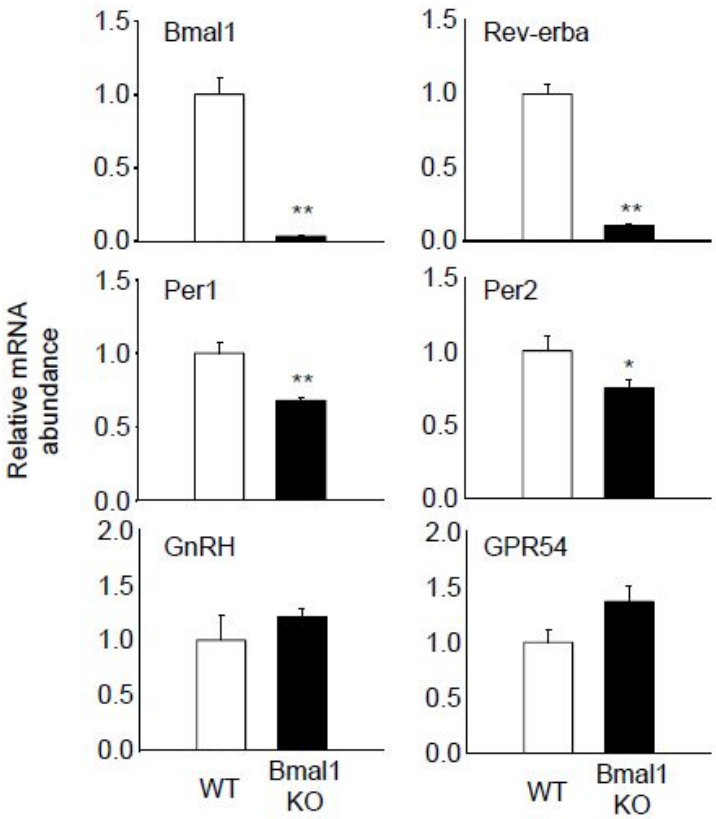

Relative mRNA abundance
Bmal1
Rev-erba
Per1
Per2
GnRH
GPR54
1.5
1.0
0.5
0.0
2.0
**
*
WT
Bmal1 KO

Figure 26. Spontaneous ultradian rhythm of GnRH expression. (A) Cultured POA slice was obtained from neonatal brain of WT or *Bmal1* KO mouse harboring GnRHp-dsLuc reporter. Bioluminescence was monitored for more than 72 hour. Asterisks indicate identified peaks. (B) Pulse period of individual GnRH neuron. (C) Pulse amplitude of individual GnRH neuron. Amplitude was normalized by mean activity under basal conditions. Data are presented as box plot (midline, median; box, 75% and 25%; error bar, 90% and 10% percentile) (n = 4 batches for each genotype).

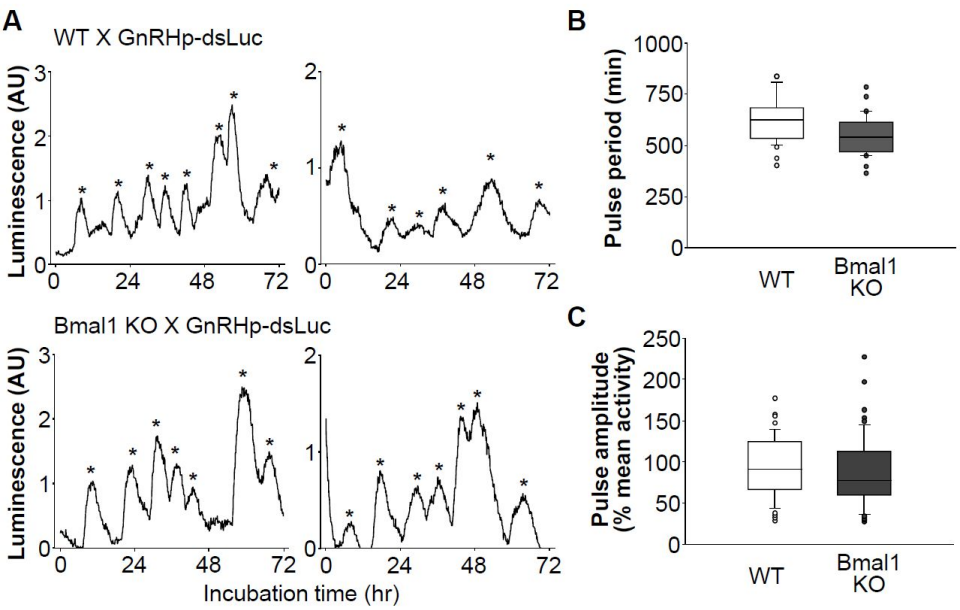

A
WT X GnRHp-dsLuc
Luminescence (AU)
Bmal1 KO X GnRHp-dsLuc
Incubation time (hr)
B
Pulse period (min)
WT
Bmal1 KO
C
Pulse amplitude (% mean activity)
WT
Bmal1 KO

Figure 27. Synchronization of GnRH expression in the *Bmal1* knockout mouse under basal conditions. (A) Synchronization of GnRH neurons in either WT or *Bmal1* KO slices. Synchronization was shown as percentage of neurons simultaneously reaching peak. (B) Profile of GnRH secretion under basal conditions. Data are shown as the mean ± s.e.m. Asterisks indicate identified peak (n = 3 for each genotype).

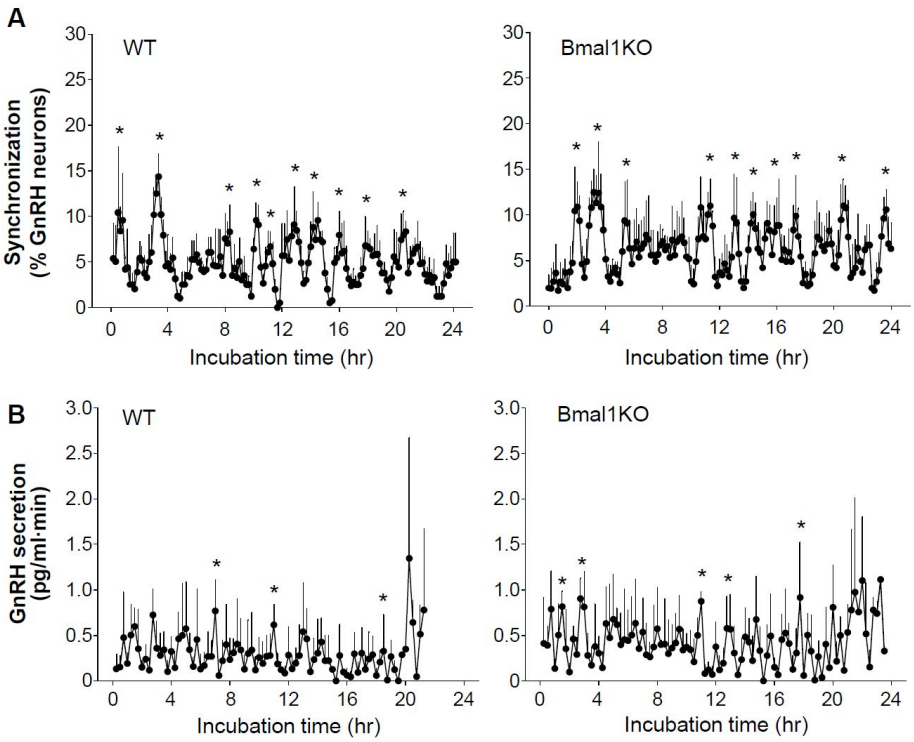

A
WT
Bmal1KO
Synchronization
(% GnRH neurons)
Incubation time (hr)
B
WT
Bmal1KO
GnRH secretion
(pg/ml·min)
Incubation time (hr)

Figure 28. Kisspeptin-induced synchronous GnRH expression in *Bmal1* knockout mouse. (A, B) Representative profiles of an individual GnRH neuron applied with intermittent kisspeptin administration (KISS: kisspeptin, 10 nM) in a pulsatile manner (15-min on: 45-min off). Blue bar, pulse of indicated stimulation. Data are shown as raw data (A) or the detrended profile (B). (C) Heatmap plot of normalized detrended luminescence for a representative batch. Each row represents an individual GnRH neuron (green, low; red, high). Synchronization index are shown as the mean ± s.e.m (n = 3-4 batches for each treatment). Gray bars indicate typical range of synchronization reached under basal conditions.

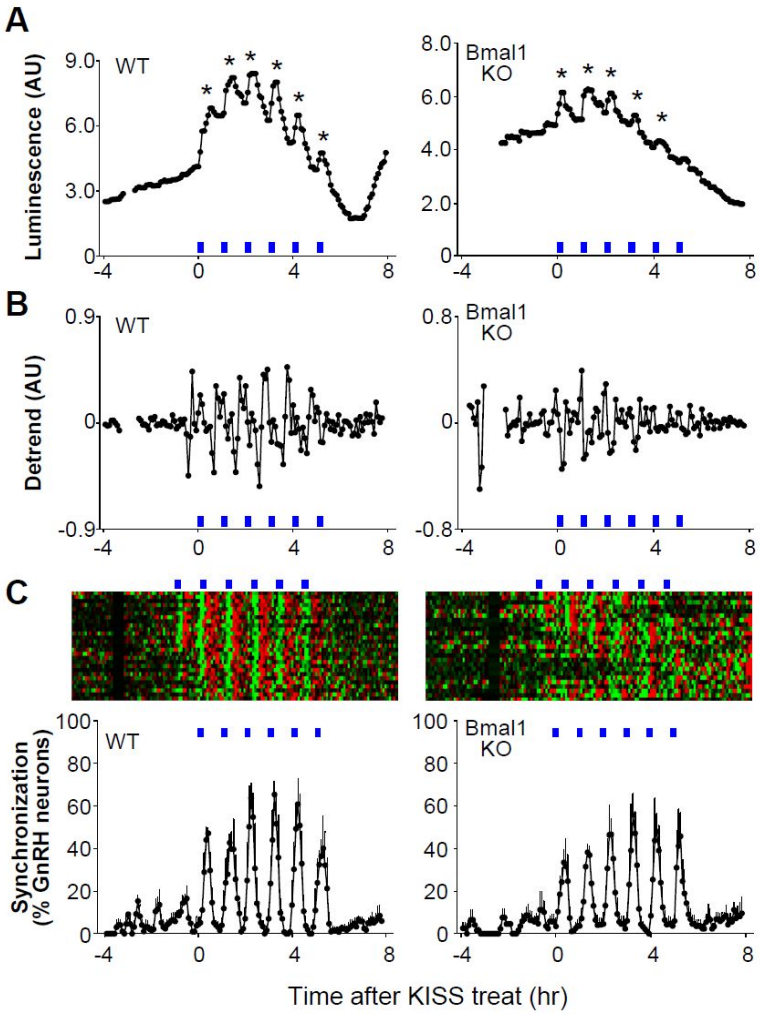

A
Luminescence (AU)
WT
Bmal1
KO
B
Detrend (AU)
C
Synchronization
(% GnRH neurons)
Time after KISS treat (hr)

Figure 29. An intermittent administration of kisspeptin-induced GnRH secretion from the POA slice. (A) Pulsatile GnRH secretion from the POA slice derived from either WT or *Bmal1* KO mouse. (B, C) Maximum value of synchronization (B) and GnRH secretion (C). (D) Cumulative GnRH secretion during 6-hr kisspeptin treatment. Data are presented as mean ± s.e.m (n = 3-4 batches for each genotypes).

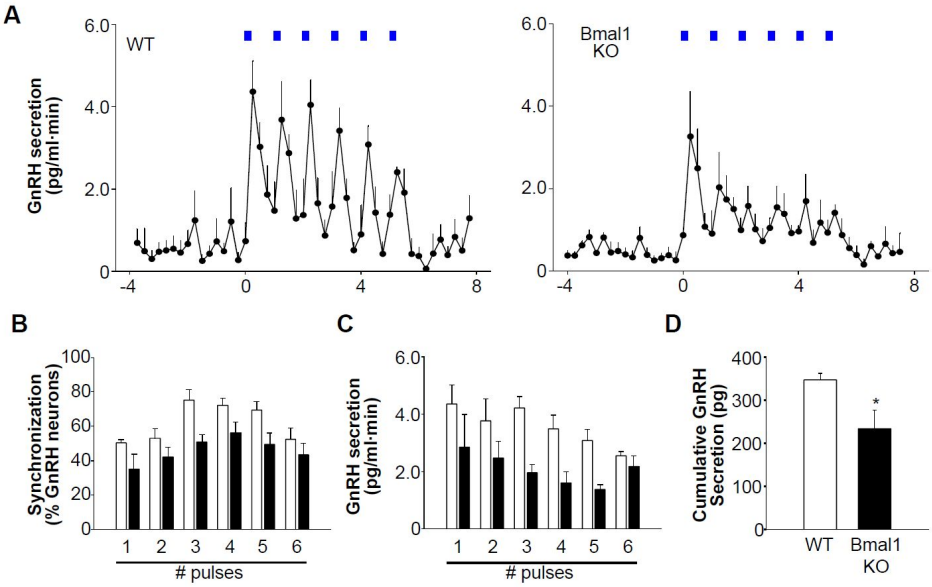

A
WT
Bmal1
KO
GnRH secretion
(pg/ml·min)
6.0
4.0
2.0
0
-4
0
4
8
B
Synchronization
(% GnRH neurons)
100
80
60
40
20
0
1 2 3 4 5 6
pulses
C
GnRH secretion
(pg/ml·min)
6.0
4.0
2.0
0
1 2 3 4 5 6
pulses
D
Cumulative GnRH
Secretion (pg)
400
300
200
100
0
*
WT
Bmal1
KO

Figure 30. Response of GnRH neuron to continuous administration of kisspeptin in Bmal1 knockout mouse. (A, B) Representative profiles of an individual GnRH neuron in slice derived from WT or *Bmal1* KO. Each slice was applied with kisspeptin (2.5 nM) for 6 hr. Blue bar, pulse of indicated stimulation. Data are shown as raw data (A) or the detrended profile (B). (C) Heatmap plot of normalized detrended luminescence for a representative batch. Each row represents an individual GnRH neuron (green, low; red, high). Gray bars indicate typical range of synchronization reached under basal conditions. (D) Profile of GnRH secretion with application of kisspeptin. Data are shown as the mean ± s.e.m (n = 3-4 batches for each genotype).

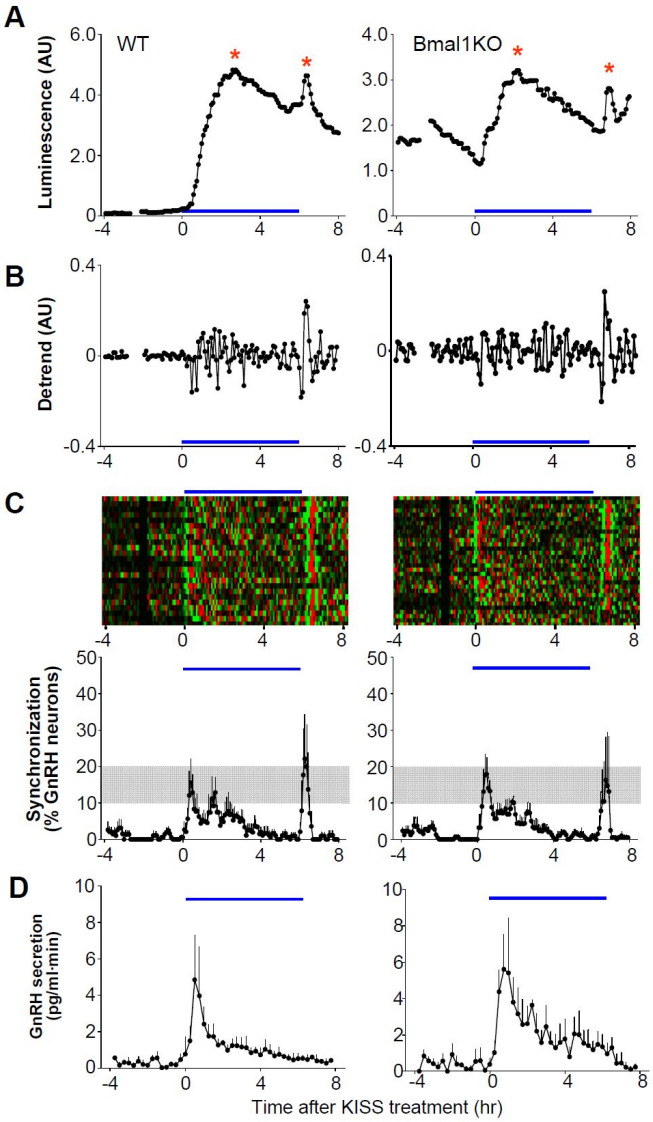
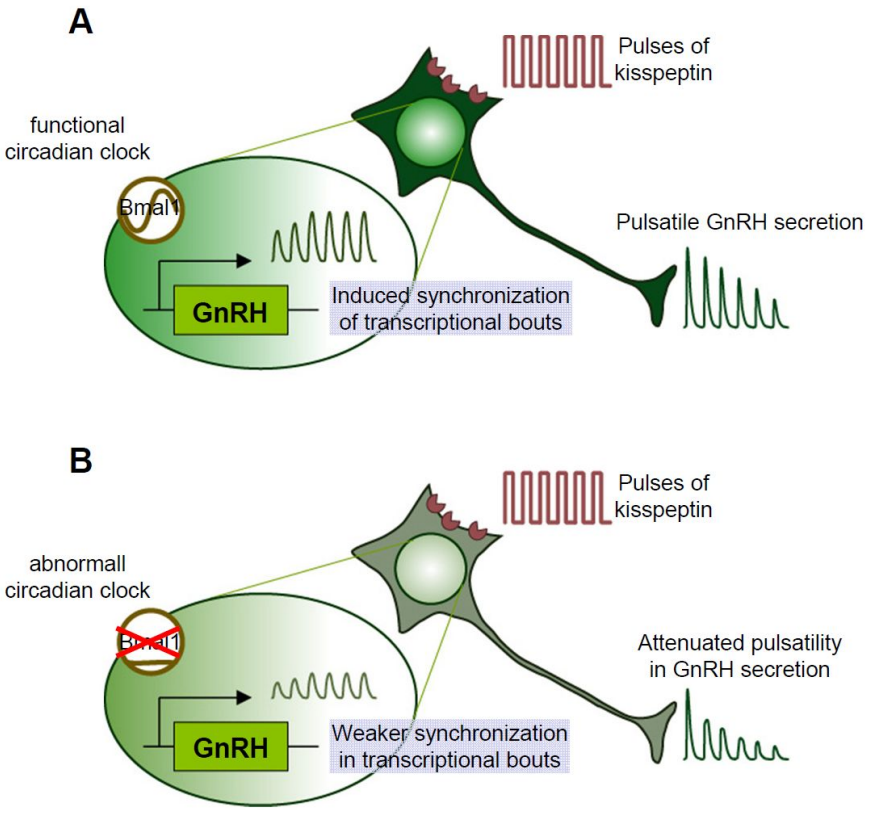

A
WT
Bmal1KO
Luminescence (AU)
B
Detrend (AU)
C
Synchronization (% GnRH neurons)
D
GnRH secretion (pg/ml·min)
Time after KISS treatment (hr)

Figure 31. A representative diagram of kisspeptin-induced GnRH pulse generation in *Bmal1* KO mouse. (A) In wildtype mouse harboring functional circadian clock, pulses of kisspeptin induces synchronization of the GnRH neurons by producing transient bouts in the promoter activity. Kisspeptin stimulation also reinforces the pulsatile GnRH secretion, thereby associating the synchronization of GnRH gene expression and secretion. (B) In *Bmal1* mouse lacking functional circadian clock, pulses of kisspeptin is capable to induce only weak level of synchronization in GnRH transcription. Reinforcement of pulsatile GnRH secretion is markedly attenuated, suggesting that circadian clockwork mediates a proper induction of GnRH pulsatility by kisspeptin.


A
Pulses of
kisspeptin
functional
circadian clock
Bmal1
Pulsatile GnRH secretion
GnRH
Induced synchronization
of transcriptional bouts
B
Pulses of
kisspeptin
abnormall
circadian clock
Attenuated pulsatility
in GnRH secretion
GnRH
Weaker synchronization
in transcriptional bouts

DISCUSSION

With recent progress in chronobiological researches, various levels of biological rhythms have been studied (Goldbeter, 2008). Hierarchical interaction between different forms of biological rhythms seems to be a very important question. The present study elucidates a possible role of molecular clockwork in spontaneous and kisspeptin-evoked GnRH pulse generation. As shown in Fig. 28 and 29, organotypic POA slice culture derived from GnRHp-dsLuc X *Bmal1* KO crossbred mouse exhibited altered responses to the intermittent kisspeptin stimulation in terms of synchronization despite no apparent differences in spontaneous GnRH gene transcription. Normal response to continuous kisspeptin administration suggested that suppressed pulsatility in GnRH secretion in *Bmal1* KO animal was not simply originated from lack of kisspeptin responsibility, but probably through the processing, storage, or secretion of the GnRH peptide as well as synchronization of GnRH neuronal population (Fig. 31).

While accumulating evidence suggests that circadian clockwork appears to mediate the normal function of reproductive system (Miller et al., 2004; Boden et al., 2010), the site of clock gene action remains relatively unclear. It has been argued earlier that cellular circadian clock machinery may be required for spontaneous GnRH pulsatility in GT1 cell line (Chappell et al., 2003). However, the character of hypothalamic GnRH neurons with genetic ablation of circadian clock genes has never been explored. In addition,

widespread effect of clock gene disruption would compound the issue. For example, circadian clockwork orchestrates steroidogenesis with StAR, a rate-limiting enzyme, as a molecular linker (Son et al., 2008), thereby the production and release of sex steroids are affected by the genetic ablation of *Bmal1* (Boden et al., 2010). In the present study, I focused on the hypothalamic GnRH neurons obtained from neonatal POA slice devoid of SCN and matured *ex vivo* (Fig. 6), which may relieve the GnRH neurons from complex effect of internal steroid milieu. Therefore, the attenuation in kisspeptin-induced pulsatility (Fig. 28 and 29) is likely to be attributable for the abnormal peripheral clockwork in the POA slices, suggesting that hypothalamic GnRH neuronal network is influenced by multiple mechanisms.

In this regards, hypothalamic brain slices with intact neuronal circuit is required to further elucidate the regulation of the GnRH pulse generator. For example, sagittal slice containing both POA and SCN would provide a valuable model to evaluate the impact of master clock on the GnRH neurons. Similarly, sagittal slice containing either AVPV or ARC in addition to POA may contain intact neuronal circuit between Kisspeptinergic and GnRH neurons.

Expression of GPR54 mRNA analyzed by real-time RT-PCR (Fig. 25) in addition to similar response to continuous administration of kisspeptin (Fig. 30) suggested that other possibility than lack of GPR54 expression may mediate the attenuated pulsatility in Bmal1 null mice. Nonetheless, kisspeptin-GPR54 system has been implicated in the circadian regulation

on the activity of GnRH neurons. It has been reported that the circadian regulation of kisspeptinergic neurons innervated from SCN (Smarr et al., 2012) and transcriptional activation by DBP (Xu et al., 2011) may play a role in the preovulatory luteinizing hormone (LH) surge (Willliam et al., 2011). Responsiveness of GnRH neuron to kisspeptin has been also suggested to be under circadian control (William et al., 2011; Zhao and Kriegsfeld, 2009) probably mediated by rhythmic regulation of GPR54 expression at the transcriptional level (Tonsfeldt et al., 2011). Therefore the regulation of GnRH neurons mediated by kisspeptin-GPR54 appears to be exploited under certain physiological occasions.

Considering that microarray studies have revealed circadian oscillations in 10-50% of total transcripts (Panda et al., 2002; Storch et al., 2002), circadian clock may play a pivotal role in the expression of key genes driving other levels of biological rhythms. Nonetheless, implication of ultradian oscillators in mediating circadian rhythm remains unknown largely because molecular identity of ultradian rhythm remains unexplored. Investigations on the ultradian biological rhythm will further provide insight into the hierarchical interaction between biological rhythms.

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국문 초록

최근 십여 년 간의 많은 연구로 생명체에 내재적인 일주기 리듬을 조절하는 분자 진자의 정체가 상당 부분 밝혀졌다. 그럼에도 불구하고 아일주기 리듬(ultradian rhythm)의 분자세포생물학적 정체에 대해서는 거의 연구된 바 없다. 따라서 본 연구는 아일주기 생체리듬의 대표적 사례로서 생식소 자극호르몬 방출 호르몬의 맥동 발진기(gonadotropin-releasing hormone (GnRH) pulse generator)를 연구하고자 한다. 1970년대 이래 GnRH 호르몬이 약 한 시간의 주기로 분비됨이 알려졌음에도 불구하고, 아직까지 GnRH 맥동 발진기의 기작은 규명된 바 없다. 본 연구의 제 1장에서는 GnRH 유전자 발현의 역동성이라는 관점에서 GnRH 맥동 발진기의 아일주기 리듬에 접근하고자 한다. 이를 위해 갓난 GnRHp-dsLuc 유전자 조작 생쥐의 시상하부 전시각 영역(preoptic area, POA)을 절편 배양하고, 이를 이용하여 단일 신경세포 수준에서 GnRH 유전자 발현을 실시간으로 관측하였다. 나아가 최근 GnRH 뉴런의 강력한 조절자로 알려진 kisspeptin에 의한 조절에 강조점을 두었다. 본 연구의 제 2장에서는 일주기 진자와 아일주기 리듬간의 연결고리를 찾고자, 분자 생체 시계의 문제가 GnRH 맥동 발진기에 미치는 영향에 주목하였다. 이를 위해 생체 시계의 핵심 전사 인자인 Bmal1이 유전적으로 결핍된 유전자 적중 생쥐로부터 얻은 GnRH 뉴런에서 맥동성을 연구하였다.

1. 제 1장에서는 GnRH 유전자 발현의 역동성을 그의 분비 패턴과 더불어 연구하였다. GnRH 프로모터 활성과 분비를 동시에 관측하기 위해 갓난 GnRHp-dsLuc 생쥐에서 POA 절편 배양을 마련하였다. 실시간 생체인광 영상 실험을 통해 기저 상태에서 GnRH 프로모터 활성이 약 10시간 주기의 아일주기 리듬을 가짐을 알게 되었다. 전체 GnRH 뉴런군에서 동기화 정도는, 비록 GnRH 분비와는 부분적으로만 연관되나, 생체 내에서 GnRH 맥동주기와 비교적 유사한 약 2시간 정도의 간격을 보였다. GnRH 뉴런을 강하게 자극하는 것으로 알려진 kisspeptin을 반복적으로 처리하였을 때에는 GnRH 프로모터 활성이 매 kisspeptin 처리에 따라 일시적으로 증가하며 맥동적 GnRH 분비 또한 크게 강화되었다. 상기의 결과들을 통해 맥동적인 kisspeptin 자극이 GnRH 전사와 분비의 시간적 패턴을 연관시킴으로써 GnRH 맥동 발진기의 조절기 역할을 수행할 것으로 사료된다.

2. 제 2장에서는 일주기 리듬과 아일주기 리듬간의 위계적인 상호작용을 연구하였다. 일주기 분자기구가 결여된 모델에서 GnRH 맥동성을 연구하고자 Bmal1 유전자 적중 생쥐로부터 POA 절편 배양을 마련하였다. Bmal1 유전자가 결여된 POA 절편 배양은 GnRH 뉴런의 수라든지 GnRH 호르몬의 함량에 있어 자연형 유래의 POA 절편 배양과 큰 차이를 보이지 않았다. GnRH 발현의 자발적인 아일주기 리듬은 Bmal1 유전자가 없이도 유지되었으나, kisspeptin에 의해 유도된 맥동성은 특히 그 분비 수준에 있어 상당한 감쇠를 보였다. 한 편 Bmal1 결여 배양에 지속적인 kisspeptin 처리시 자연형 수준의 반응을

보였는데, 이는 주기적 kisspeptin 자극에 의한 GnRH 맥동성 감쇠가 수용체 혹은 그 하위 단계의 단순한 반응성 감소에 기인하지 않음을 시사한다. 종합해 보건대, 세포 일주기 시계는 GnRH 뉴런군의 동기화뿐 아니라 GnRH 펩타이드의 가공, 저장 혹은 분비 과정을 매개함으로써 아일주기 리듬 형성에 기여하는 것으로 사료된다.

Key words: gonadotropin-releasing hormone (GnRH), pulse generator, ultradian rhythm, kisspeptin, gene expression, episodic secretion, circadian rhythm, hierarchical interaction, Bmal1

감사의 글

동경해 마지않던 자리에 서게 되면 어떤 소회가 떠오를지 궁금했었습니다. 막상 오늘이 되고서 보니 부족한 사람이 지금에 이를 수 있어 다행하다는 것과, 그 과정에서 많은 도움 주셨던 감사한 인연들이 먼저 생각납니다. 감사의 글을 적을 수 있는 자리에 올 수 있어 더 없이 행복한 마음입니다.

늘 부족한 제자를 항상 이해해 주시고 기다려 주셨던 멘토, 김경진 교수님께 무엇보다도 큰 감사의 말씀을 드리고 싶습니다. 선생님께서 주셨던 교훈을 토대로 인생과 과학을 대하는 철학에 대해 많이 배울 수 있어 영광이었습니다. 아울러 바쁘신 중에도 졸고를 심사해 주시고, 고견으로써 이끌어 주신 정진하 교수님, 이건수 교수님, 최석우 교수님, 성재영 교수님께도 진심으로 감사드립니다. 파이펫 쥐는 것부터 논문 쓰는 법, 나아가 인생의 많은 것들을 가르쳐 주신 사수 손기훈 교수님께도 깊이 감사드립니다. 제가 위기의 상황에 처했을 때도, 늘 자상히 배려해 주시고 많은 가르침 주신 정수영 박사님께도 역시 깊은 감사 인사 드립니다. 항상 차분하게 격려해 주셨던 김희대 박사님께도, 함께 학위를 준비하는 과정에서 페이스 메이커 역할을 해 준 이은정 박사에게도 진심 어린 감사를 표하고 싶습니다. 서로 바쁜 와중에도 궂은 일들 묵묵히 도맡아 준 승국과 성식에게도, 입학하자 마자 정신없는 선배 따라다닌 정아와 도연에게도 고맙고 미안하단 말을

전하고 싶습니다. 구체적으로 언급하지 못한 실험실 선후배, 동료 여러분께도 양해를 표하며, 감사의 마음을 전합니다.

제멋대로인 아들이지만, 뜻하는 바를 추구할 수 있도록 늘 성원해주신 부모님께도 깊이 감사드립니다. 말로는 잘 표현하지 않았지만, 사랑합니다. 객지에 있는 조카를 위해 늦은 시간에도 많이 달려와 주신 이모님, 이모부님, 충민이에게도 진심 어린 감사의 말씀을 드립니다.

오늘의 배운 바를 토대로 하여, 어떻게 살아야 하고 어떻게 연구해야 할 지를 부족하나마 늘 고민하는 사람이 되도록 하겠습니다. 감사합니다.