이학박사학위논문

Kisspeptin 신경세포 특이적 칼슘 활성과 GnRH 맥동 발진기에 관한 연구

Studies on kisspeptin neuron-specific calcium oscillation in the arcuate nucleus and the GnRH pulse generator

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ABSTRACT

Pulsatile secretion of gonadotropin-releasing hormone (GnRH) is crucial for initiating and maintaining development and reproduction. Kisspeptin neurons in the hypothalamic arcuate nucleus (ARN) appear to be essential components that control GnRH neurons, the final common pathway for central regulation of mammalian reproduction. Since the discovery of GnRH 40 years ago, considerable amount of studies on the GnRH pulse has been conducted in the field of neuroendocrinology. However, the underlying mechanisms of the GnRH pulse generator and the contribution of kisspeptin neurons are still not fully understood. Therefore, the present study aimed to examine whether ARN kisspeptin neurons can generate pulsatility. To address this, I examined the endogenous rhythmicity of ARN kisspeptin neurons and its neural regulation. Calcium dynamics from the population of individual ARN kisspeptin neurons was monitored from neonatal organotypic slice cultures of *Kiss1*-IRES-Cre mice transduced with genetically encoded calcium indicators, by using a real-time imaging device. Pharmacological approaches were employed to examine the effects of voltage-gated ion channel blockers, neuropeptides released from ARN kisspeptin neurons, and NMDA and GABA receptor-mediated neurotransmission inhibition. Chemogenetic approach to manipulate kisspeptin neuron-specific activity was also utilized to assess the contribution of ARN kisspeptin neurons on the

population dynamics. Lastly, the relation with the GnRH pulse generator was examined using organotypic slice cultures of GnRH promoter-driven luciferase-expressing mice. ARN kisspeptin neurons exhibited a robust and synchronized calcium oscillation *ex vivo*, in an ultradian cycle with a period of approximately 3 min, in contrast to the irregular and desynchronized oscillations in pan-neuronal populations. Kisspeptin neuron-specific calcium oscillations were dependent on action potential-mediated synaptic transmission, marginally influenced by autocrine actions of the neuropeptides, and regulated by NMDA and GABA receptor-mediated neurotransmission. In the organotypic co-culture containing brain slices with kisspeptin and GnRH neurons, GnRH gene expression, which could be induced with exogenous kisspeptin application, was partly diminished by pharmacological blockade of kisspeptin receptor signaling. The present study demonstrated that ARN kisspeptin neurons are capable of generating synchronized and self-sustained calcium oscillation *ex vivo*. These calcium oscillation was regulated by multiple factors within the ARN region, including the kisspeptin neurons themselves. Moreover, the present study suggests that kisspeptin signaling have a certain role in maintaining basal GnRH gene transcription, and contributes to the GnRH pulse generator.

Key words: kisspeptin, arcuate nucleus, calcium oscillation, ultradian rhythm, gonadotropin-releasing hormone (GnRH), pulse generator, synchronization

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BACKGROUND AND PURPOSE

BACKGROUND

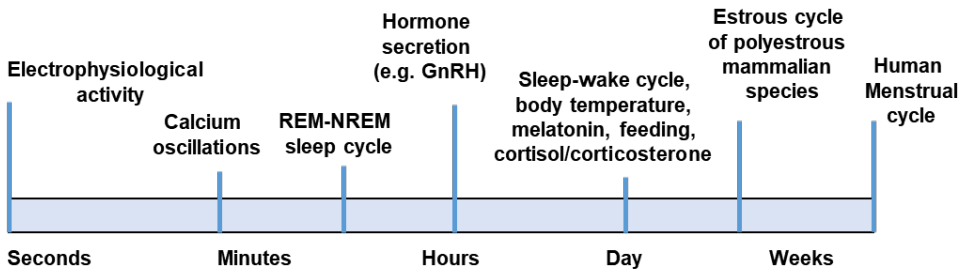
1. Biological rhythms

1.1 Biological oscillations in physiological systems

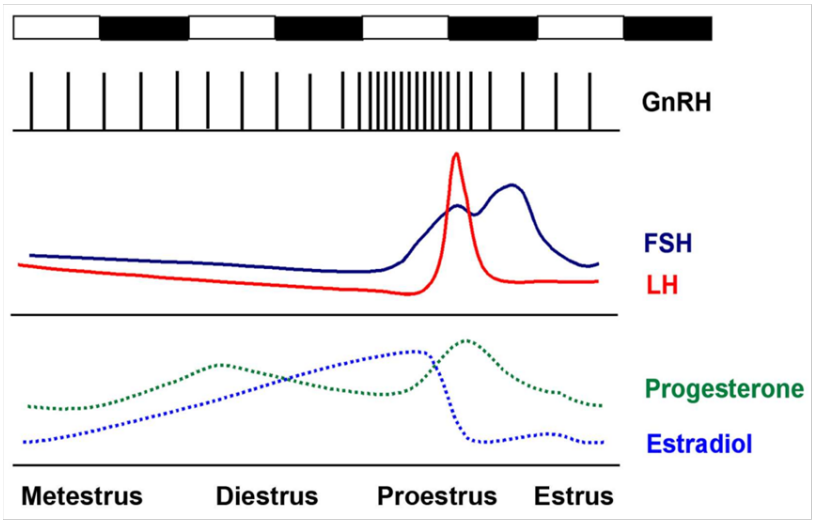
Numerous physiological systems from bacteria to human follow various levels of biological oscillations. These oscillations include circadian rhythm which occurs with a period of approximately 24 hr, infradian rhythm which is longer than 24 hr, and ultradian rhythm which occurs multiple times within 24 hr (Fig. 1) (Dibner et al., 2010; McGinnis and Young, 2016). Infradian rhythms include reproductive cycles, which range from 4 to 5 days in rodents, 28 days in humans, to a season-dependent annual cycle in sheep (Bartlewski et al., 2011; Plant, 2015). In the mouse, ovulation is controlled by circadian regulation. When estradiol levels peak, timing signal from the central circadian pacemaker is required to induce the surge of gonadotropin releasing hormone (GnRH) release (Fig. 1A). Also, dysregulation of circadian rhythm affects the length and progression of the estrus cycle and the establishment and maintenance of pregnancy (Miller and Takahashi, 2013). Thus, biological rhythms can affect many physiological systems in a process by which ultradian, circadian, and infradian rhythm are related to each other. From the generation of action potentials in neurons, alternation of day and

Figure 1. Various types of biological rhythms. (A) Diverse biological rhythms exist in various levels. Action potentials in neurons occur in seconds range and intracellular calcium oscillations with minutes' range. The gonadotropin-releasing hormone (GnRH) are secreted in hours' scale. (B) Biological rhythms can affect many physiological systems in which ultradian, circadian, and infradian rhythm are related to each other. The mouse ovulation, which cycles every 4 to 5 days, is controlled by circadian regulation. When estradiol levels peak, timing signal from the central circadian pacemaker is required to induce the surge of GnRH release and subsequent luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Image obtained from Miller and Takahashi, 2014, Front Endocrinol. (C). Ultradian rhythms are often found in relation with circadian rhythm. Sleep, although considered as a circadian phenomenon, is composed of ultradian rhythm of rapid eye movement (REM)/non-REM (NREM) sleep cycles that have a period of about 90 min. Image obtained from LucidDreamExplorers.com

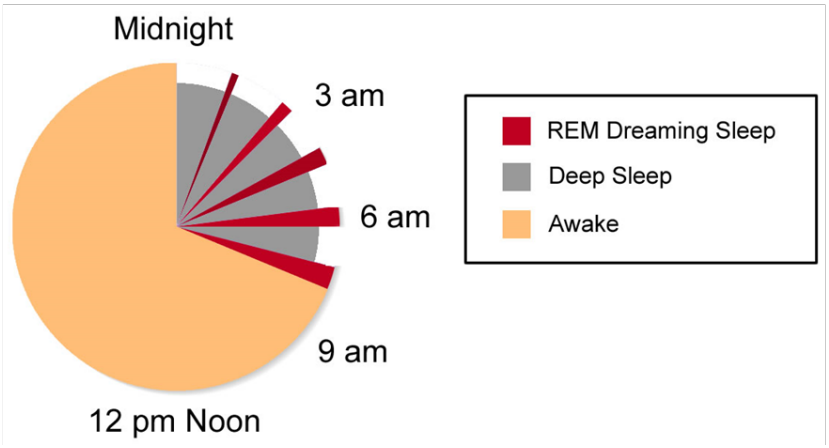
A


Electrophysiological activity
Calcium oscillations
REM-NREM sleep cycle
Hormone secretion (e.g. GnRH)
Sleep-wake cycle, body temperature, melatonin, feeding, cortisol/corticosterone
Estrous cycle of polyestrous mammalian species
Human Menstrual cycle
Seconds
Minutes
Hours
Day
Weeks

B


GnRH
FSH
LH
Progesterone
Estradiol
Metestrus
Diestrus
Proestrus
Estrus

C


Midnight
3 am
6 am
9 am
12 pm Noon
REM Dreaming Sleep
Deep Sleep
Awake

night, and to the cycle of seasons, numerous processes in natural life possess repetitive and rhythmic oscillations with a specified period (Goldbeter, 2008). These biological oscillations are more likely detected when synchronized among the population. Some biological rhythms are regulated by self-sustained oscillators that are maintained in the absence of environmental cues, while others are controlled by external signals (Gachon et al., 2004).

The most characterized biological rhythms so far are circadian rhythms. Circadian rhythm is considered as an evolutionary adaptation of the organisms to the daily environmental cycles resulted from the rotation of the

Earth. Most organisms have internal timekeeping system that allows the organism to adjust to daily changes of the environment. In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus functions as the central pacemaker for circadian rhythm (Hastings et al., 2018). The Nobel Prize in Physiology or Medicine 2017 was awarded in recognition of the discovery of circadian molecular clock composed of clock genes in Drosophila. The molecular machinery of the circadian clock is present in many species including plants, animals, fungi, and cyanobacteria (Edgar et al., 2012). In mammals, clock genes form a transcriptional-translational feedback loop comprised of activators including CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1), and repressors including PER (period) and CRY (cryptochrome), controlling rhythmicity of their own expression (Takahashi, 2017). The resulting oscillations are reflected from the level of behavior to

gene expression (Merrow et al., 2005).

The mammalian circadian system is composed of multiple oscillators organized in a hierarchy. The central pacemaker in the SCN receives external signals, or zeitgebers, every day by light stimulation through the retinohypothalamic tract and orchestrates clocks in the periphery (Stratmann and Schibler, 2006). The peripheral tissues also contain clocks capable of oscillating on their own, as shown in immortalized rat-1 fibroblasts that expressed circadian gene expression induced by serum shock (Balsalobre et al., 1988). Peripheral clocks exist in various organs including liver, heart, and intestine, and zeitgebers other than light, such as food intake, could also play a role in peripheral clock reset (Richards and Gumz, 2012).

Disruption of the circadian rhythm is related in metabolic processes and abnormal brain function. Hormones involved in metabolism exhibit circadian oscillation, and in particular, leptin, an adipocyte-derived circulating hormone that suppresses appetite and increases metabolism, depends on the SCN to maintain its circadian rhythmicity in rodents. Also, circadian rhythmicity of insulin secretion and glucose tolerance disappeared in type 2 diabetes patients (Froy, 2010). Similarly, abnormal sleep and circadian rhythm dysfunction are frequently observed in patients with psychiatric disorders and neurodegenerative diseases. Changes in sleep profile are reported in patients who suffer from depression, and irregular sleep timing and reduction of total sleep time trigger manic episodes in bipolar disorder. One of the features of Alzheimer’s disease includes fragmented night-time sleep and sundowning

syndrome, a tendency to be confused and agitated in the late afternoon and evening. Also, more than 80% of Parkinson's disease patients have sleep disorders and disruption of basic rapid eye movement (REM) and non-REM (NREM) sleep architecture (Wulff et al., 2010).

1.2 Ultradian rhythm and synchronization

Ultradian rhythms refer to biological oscillations that occur multiple times throughout a day. Ultradian rhythms are often found in relation with circadian rhythm. Sleep, although considered as a circadian phenomenon, is composed of ultradian rhythm of REM/NREM sleep cycles that have a period of about 90 min (Fig. 1B) (Lamont and Amir, 2017). Ultradian sleep cycles are established during early childhood, and the duration of the ultradian REM/NREM cycle increase with age to a more adult-like pattern (Lopp et al., 2017). Synchronized calcium signals with an ultradian rhythm in hypothalamic slices of the subparaventricular zone and paraventricular nucleus (SPZ-PVN) superimpose on the circadian fluctuation of calcium signals in the SCN (Wu et al., 2018). Also in the pattern of glucocorticoid secretion, an hourly ultradian pulsatility exists together with circadian rhythm, to maintain stress responsivity and prevent downregulation of glucocorticoid signaling systems (Lightman and Conway-Campbell, 2010).

Ultradian rhythm is also present in many other phenomena. In the developmental stages, the expression of transcription factors such as Ascl1

and Hes1, oscillate to maintain proliferating neural progenitor cells and sustained expression promotes neuronal fate determination (Imayoshi et al., 2013); additionally, Wnt and Notch signaling pathway oscillations are critical for the segmentation of mouse embryonic mesoderm (Sonnen et al., 2018). Gene expression also oscillates with ultradian rhythmicity, and the rhythm is more apparent when the ultradian timing system contributes more to overall biological timing (van der Veen and Gerkema, 2017). Pulsatile release of GnRH plays an essential role in the development of sexual function and proper regulation of the reproductive cycle, in contrast to the surge GnRH release for ovulation (Herbison, 2016).

In addition, synchronized pattern of ultradian rhythm exert diverse physiological functions. Oxytocin neurons in the hypothalamus display synchronized bursting electrical activity during lactation, every 3 to 15 min to induce milk ejection (Israel et al., 2003). Also, synchronized activity of interneurons are able to generate gamma frequency oscillations in cortical neurons (Mishra et al., 2006), and the synchronization pattern can determine the form of neuronal interaction (Womelsdorf et al., 2007). Although ultradian rhythms exist in numerous biological functions, their characteristics remain unclear.

1.3 Calcium oscillations in neurons

Calcium oscillations in cells can represent signals to transmit

biological information (Smedler and Uhlen, 2014). Monitoring changes in intracellular calcium concentration using genetically encoded calcium indicators offers access to cell type-specific calcium dynamics to characterize neuronal physiology (Grienberger and Konnerth, 2012). GnRH neurons in cell culture or acute brain slices display spontaneous calcium transients that correlate with their burst firing pattern (Jasoni et al., 2010; Terasawa et al., 1999). Recently, calcium imaging was utilized to observe ultradian rhythms in hypothalamic slices of SPZ-PVN that was superimposed to the circadian rhythm in the SCN (Wu et al., 2018).

Calcium imaging uses indicators to observe the change in intracellular calcium levels. There are chemical indicators such as Fura-2 or Calcium-Green which are small molecules that have to enter the cell to chelate calcium ions. The first real-time calcium imaging was carried out with these chemical indicators in cardiac cells (Cannell et al., 1987). Also, there are genetically encoded calcium indicators (GECIs) in which genes encoding the proteins can be transfected (Nakai et al., 2001). They are composed of calmodulin, M13 domain of the myosin light chain kinase, and fluorescent proteins which undergo conformation change in the presence of calcium. GCaMP and RGECO are widely used GECIs that are used as neuronal activity readout. For example, in the brain slice of the SCN, the control center of circadian rhythm, calcium oscillations nicely reflected the robustness of the neuronal activity, along with the bioluminescence expressed by the clock gene promoters (Brancaccio et al., 2019; Brancaccio et al., 2017).

Intracellular calcium signaling can also provide information on the state of the cell since dysregulation of neuronal calcium homeostasis is related to the pathogenesis of Alzheimer's disease (Tong et al., 2018). Also, massive intracellular calcium influx triggers or modifies excitotoxic necrosis and apoptosis, suggesting that changes in calcium levels affect the two death pathways (Zipfel et al., 2000). Likewise, calcium homeostasis undergoes dysregulation in physiological aging along with oxidative stress, causing changes in calcium-dependent cell excitability, synaptic plasticity, and connectivity (Wojda et al., 2008). Particularly in neurons, calcium regulates neuronal plasticity and synaptic transmission, and the amplitude and duration of calcium transients are crucial in determining synaptic plasticity (Jedrzejewska-Szmek et al., 2017). A novel optogenetic tool OptoSTIM, that can manipulate intracellular calcium levels by activating calcium-selective endogenous calcium release-activated calcium (CRAC) channels, could enhance the learning capacity of mice when used *in vivo* (Kyung et al., 2015).

2. GnRH pulse generator

2.1 Emerging discipline in neuroendocrinology

Neuroendocrinology, as the name implies, refers to the field that studies the interface between the nervous system and the endocrine system. Geoffrey W. Harris is considered as the father of neuroendocrinology. His

‘neurohumoral hypothesis’ presented that the hypothalamus secretes hypothalamic releasing factors (RF) through the hypophyseal portal system, the blood vessels between the hypothalamus and the pituitary (Harris, 1948). His pioneering research established the founding for the Nobel prize in Physiology or Medicine, in 1977, which was shared by three scientists who discovered the structure and function of GnRH, and the development of radioimmunoassay that can detect the levels of peptide hormones. Soon after its discovery, GnRH was modified to create various modulators of GnRH receptor signaling. Many agonists and antagonists were developed for medication to influence the function of the pituitary and gonads, to affect fertility, the capability to produce offspring.

The hypothalamus, which is the region of particular interest in neuroendocrinology, is located at the base of the brain. The hypothalamus is involved with critical functions for maintaining homeostasis such as energy metabolism, body temperature, autonomic function, circadian rhythms, and reproduction (McCartney and Marshall, 2014). The hypophyseal portal system provides the functional connection between the hypothalamus and anterior pituitary, or the adenohypophysis. The anterior lobe of the pituitary is a major organ in the endocrine system that regulates physiological processes including stress, growth, reproduction, and lactation. The anterior pituitary is derived from the ectoderm, and contains endocrine cells including the gonadotropes, which secrete the hormones LH and FSH.

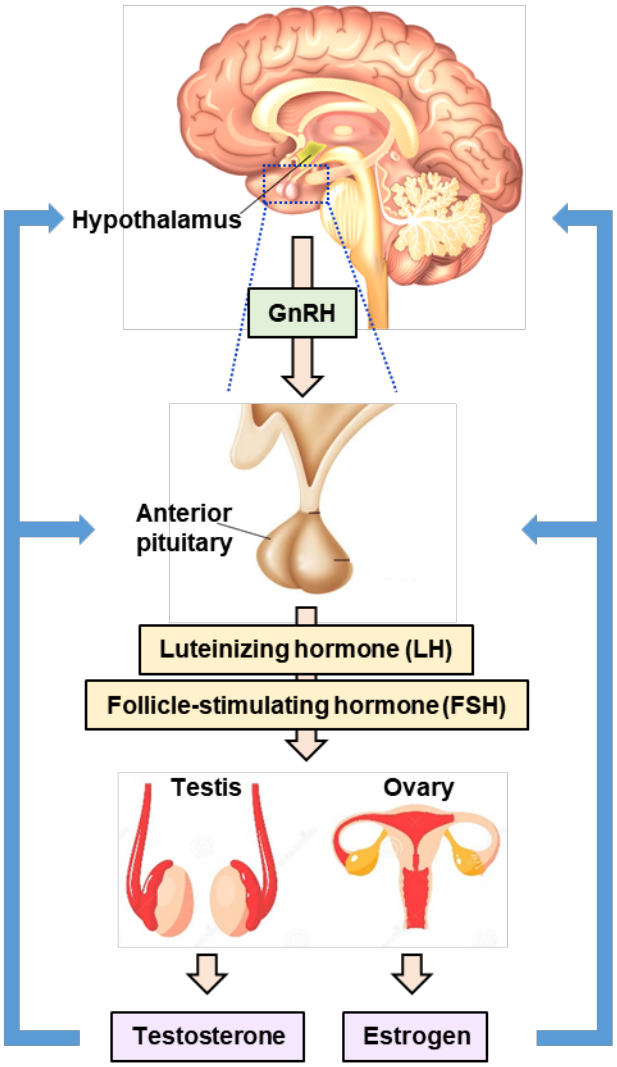
2.2 Hypothalamic-pituitary-gonadal (HPG) axis

The hypothalamic-pituitary-gonadal (HPG) axis is composed of three organs of the body. From the brain, the peptide hormone GnRH is secreted through the hypophyseal portal system to the anterior pituitary, where pituitary hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are released to the gonads. The testes or ovaries then produce testosterone or estrogen, respectively, that can feedback to the hypothalamus or the anterior pituitary (Fig. 2). The activity of the HPG axis is critical in the development and regulation of reproduction, the process in which offspring are produced. However, simply activating the axis was not sufficient. The GnRH agonists that were developed to increase gonadotropins initially stimulated the secreted hormone levels, but then caused a sustained drop. Sustained stimulation desensitizes the GnRH receptor-mediated gonadotrope secretion (Finch et al., 2009). Later, a critical study discovered the importance of the mode of GnRH secretion acting on the pituitary.

GnRH neurons in the hypothalamus have two modes of secretion. One is the surge mode that is triggered by high levels of estradiol in the late follicular phase. Estradiol acts via transcriptional processes through estrogen receptor α (ERα) to alter the activity of neurons that project and activate GnRH neurons, which sequentially leads to LH surge. The neurons responsible of inducing ovulation by stimulating LH surge with the high levels of estradiol are located in the anteroventral periventricular nucleus (AVPV) of

Figure 2. Hypothalamic-pituitary-gonadal neuroendocrine axis. The Hypothalamic-pituitary-gonadal (HPG) neuroendocrine axis is composed of three organs of the body. From the brain, the peptide hormone GnRH is secreted to the anterior pituitary, where pituitary hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are released to the gonads. The testes or ovaries then produce testosterone or estrogen, respectively, that can feedback to the hypothalamus or the anterior pituitary

Hypothalamic-pituitary-gonadal (HPG) neuroendocrine axis



the hypothalamus. The other is the pulse mode that is responsible for driving episodic LH secretion in mammalian species. The pulse activity is essential for puberty onset and is known to trigger the first ovulation in females, but the mechanism by which GnRH neurons achieve this pattern of release remains unclear.

2.3 GnRH pulse generator

GnRH pulse generator that governs pulsatile secretion of GnRH is a well-characterized ultradian rhythm important for central regulation of reproduction (Herbison, 2018; McCartney and Marshall, 2014). The importance of the GnRH pulse generator was revealed when intermittent administration of GnRH led to pulsatile gonadotropin secretion in monkeys, while constant infusion desensitized the pituitary response (Belchetz et al., 1978). Subsequently, pulsatile GnRH secretion was validated in other animals including rodents, sheep, and even humans (Herbison, 2016). Furthermore, the secretion pattern of GnRH was correlated with LH pulses, indicating that GnRH secretion generates the pulsatility (Moenter et al., 1992). These pulse patterns of GnRH are regulated by multiple factors including feedback actions of the steroids released by the ovaries or testes and the energy balance and stress within the internal environment (Moenter, 2010). Therefore, GnRH neurons are considered as the final common pathway for central regulation of mammalian reproduction. GnRH neurons exhibit

pulsatile secretion and synchronized burst activities even in immortalized cells, yet the origin of the pulse generator still remains elusive (Wetsel et al., 1992). The most widely accepted hypothesis is that kisspeptin neurons in the arcuate nucleus (ARN) are the potent drivers of the GnRH pulse generator. In this proposed model, ARN kisspeptin neurons activate the processes of GnRH neurons to generate pulsatile secretion (Fig. 3).

3. Kisspeptin neurons in the hypothalamic arcuate nucleus

3.1 Kisspeptin neurons in the hypothalamus

Kisspeptin is a neuropeptide encoded by *Kiss1* gene. It was formerly known as metastin because of its ability to suppress tumor metastasis. Now, kisspeptin neurons are considered as an important regulator of mammalian reproduction and puberty, by exerting its effect on GnRH neurons. Kisspeptin is produced from kisspeptin neurons, mainly located in the two nuclei of the hypothalamus. AVPV kisspeptin neurons are located near the POA where the cell body of GnRH neurons exist, whereas ARN kisspeptin neurons are located near the median eminence (ME) where the axon terminals of GnRH neurons reach (Fig. 4). The two populations are known to regulate different aspects of GnRH secretion. AVPV kisspeptin neurons are thought to underlie the surge mode of GnRH secretion in females in the pre-ovulatory phase while ARN kisspeptin neurons are implicated in pulsatile GnRH release which

Figure 3. GnRH pulse generator. A proposed model for ARN kisspeptin neurons modulating the GnRH pulse generator. A group of neurons in the ARN activates the distal processes of GnRH neurons to generate episodes of GnRH secretion. The pulse generator can also be modulated by external inputs including the preoptic area (POA) and the paraventricular nucleus (PVN), and others. Image obtained from obtained from Herbison, 2018, Endocrinology.

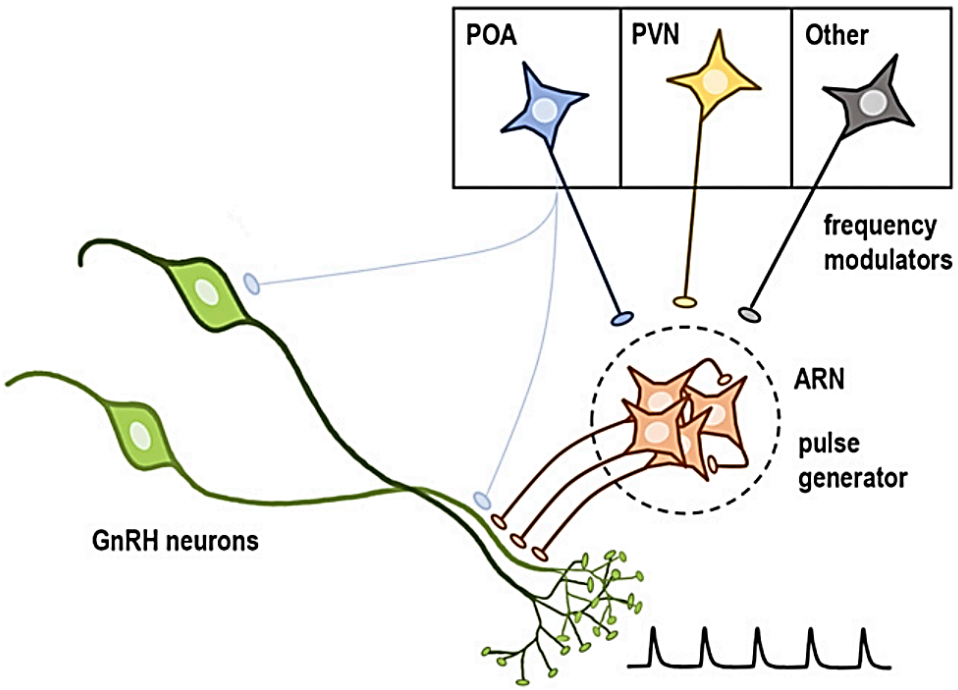
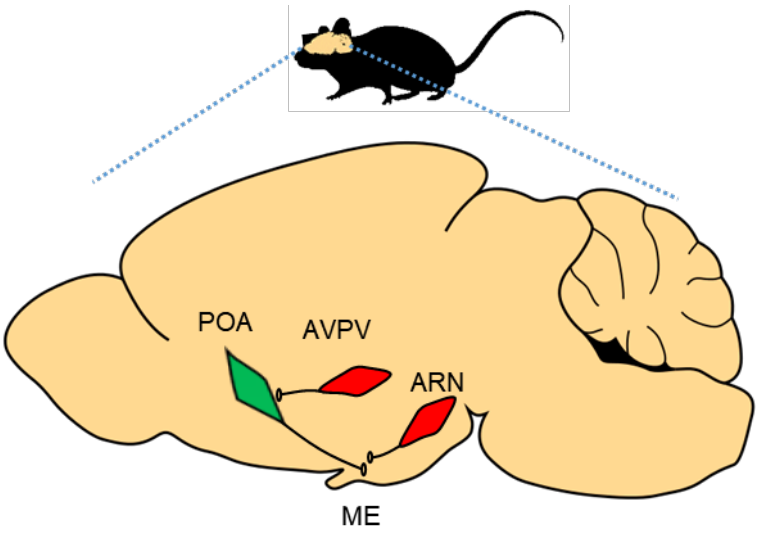

POA
PVN
Other
frequency
modulators
ARN
pulse
generator
GnRH neurons

Figure 4. Rodent hypothalamic kisspeptin and GnRH neurons. Kisspeptin neurons are mainly located in the two nuclei in the mouse hypothalamus. Anteroventral periventricular nucleus (AVPV) kisspeptin neurons are located near the preoptic area (POA) where the cell body of GnRH neurons mostly exist, whereas ARN kisspeptin neurons are located near the median eminence (ME) where the axon terminals of GnRH neurons reach.


POA
AVPV
ARN
ME

is necessary for proper reproductive function (Herbison, 2016).

Kisspeptin neurons are thought to “time” reproduction by coordinating inputs for maturation and regulation of the GnRH pulse generator (Putteeraj et al., 2016). Loss-of-function mutations in the kisspeptin receptor (Kiss1R) cause the absence of sexual maturation (Seminara et al., 2003). In addition, when secretion from the median eminence was obtained from microdialysis and measured by radioimmunoassay, the release pattern of both kisspeptin and GnRH was pulsatile, with similar timing (Keen et al., 2008).

ARN kisspeptin neurons have been shown to coordinate circadian input from the SCN and control feeding, locomotor activity, body temperature, and sleep pattern, suggesting their role in circadian regulation of physiology and behavior, in addition to fertility (Padilla et al., 2019).

3.2 ARN Kisspeptin as a potent pulse driver

Kisspeptin neurons in the ARN, located on both sides of the third ventricle, develop at embryonic day 16.5 and become synaptically connected to GnRH neurons (Kumar et al., 2015), unlike another kisspeptin population in the anteroventral periventricular nucleus (AVPV) detected at later developmental stages (Clarkson and Herbison, 2006).

Pulsatile kisspeptin administration to the preoptic area (POA) evokes synchronous GnRH promoter activity, indicating that kisspeptin inputs to GnRH neurons are important in GnRH pulse generation (Choe et al., 2013).

Also, a series of elegant studies on the driver of pulsatile GnRH activity was performed. For instance, optogenetic activation of GnRH neurons (Campos and Herbison, 2014) or the ARN kisspeptin neurons (Han et al., 2015) generated pulsatile LH secretion in both cases. In adult mice, ARN kisspeptin neurons exhibited synchronized calcium oscillations in vivo that correlated with pulsatile LH secretion (Clarkson et al., 2017). Kisspeptin neurons in the ARN are, thus, considered as an important regulator of the GnRH pulse generator (Herbison, 2018; McCartney and Marshall, 2014).

In addition, ARN kisspeptin neurons co-express neurokinin B (NKB) and dynorphin (Dyn), and are also known as KNDy neurons. NKB mostly stimulates kisspeptin neurons while Dyn mostly inhibits the activity of ARN kisspeptin neurons, alrhtough the effects are sometimes controversial (Millar, 2014; Moore et al., 2018). Nevertheless, the presence of these co-released neuropeptides suggest that ARN kisspeptin neurons may generate self-sustained oscillation through auto-regulatory feedback. However, several studies reported against kisspeptin neurons as the pulse generator. In female mice with kisspeptin neurons ablated by diphtheria toxin A, puberty onset and reproductive maturation took place, although with small ovaries (Mayer and Boehm, 2011). Another study suggest that the pulse generator for GnRH secretion involves more than one element (Ezzat et al., 2015). Therefore, it is necessary to address whether ARN kisspeptin neurons are able to generate rhythm on their own and identify the inputs that are involved in their regulation.

3.3 Calcium imaging in ARN kisspeptin neurons

When the GCaMP activity was measured using fiber photometry in adult mice, ARN kisspeptin neurons exhibited episodic calcium activity. Interestingly, the blood obtained from the tail showed pulsatile LH secretion, a surrogate marker of GnRH secretion, that correlated with the calcium episodes. These LH pulses could be modulated using optogenetics: when ARN kisspeptin neurons were activated using channelrhodopsin, LH pulses were generated, and when kisspeptin neurons were silence using archaerhodopsin, LH pulses were inhibited (Clarkson et al., 2017). These results suggest that ARN kisspeptin neurons are causal regulators of the GnRH pulse generator that can produce LH pulsatility.

Nevertheless, whether ARN kisspeptin neurons themselves can generate the rhythm has yet to be determined. Considering the controversies on kisspeptin neurons as the pulse generator, it is essential to address whether ARN kisspeptin neurons are able to generate rhythm on their own, or they simply relay neural information, and identify the inputs that are involved in their regulation.

PURPOSE

As described above, kisspeptin neurons in the hypothalamic ARN appears to be essential to regulate GnRH neurons. Although considerable amount of evidence suggests potential roles in the GnRH pulse generator, it still remains unclear how ARN kisspeptin neurons can generate pulsatility. Therefore, the present study aimed to address the following issues:

(1) Can ARN kisspeptin neurons generate ultradian calcium oscillation *ex vivo*?

(2) What are the neural signaling factors that regulate ARN kisspeptin neurons?

(3) What is the impact of the kisspeptin neurons on the GnRH pulse generator?

Synchronized calcium oscillation in the hypothalamic arcuate nucleus kisspeptin neurons *ex vivo* and the GnRH pulse generator

INTRODUCTION

Diverse physiological systems exhibit biological oscillations. These oscillations include circadian rhythm which occurs with a period of approximately 24 hr, infradian rhythm which is longer than 24 hr, and ultradian rhythm which occurs multiple times within 24 hr (Dibner et al., 2010; McGinnis and Young, 2016). Also in the pattern of glucocorticoid secretion, an hourly ultradian pulsatility exists together with circadian rhythm, to maintain stress responsivity and prevent downregulation of glucocorticoid signaling systems (Kalafatakis et al., 2016). Although ultradian rhythms exist in numerous biological functions, their characteristics remain unclear.

The hypothalamic GnRH pulse generator that governs pituitary secretion of gonadotropins is a well-characterized ultradian rhythm important for the regulation of mammalian reproduction (Herbison, 2018; McCartney and Marshall, 2014). The importance of the GnRH pulse generator was revealed when intermittent administration of GnRH led to pulsatile gonadotropin secretion in monkeys, while constant infusion desensitized the pituitary response (Belchetz et al., 1978). Subsequently, pulsatile GnRH secretion was validated in other animals including rodents, sheep, and even humans (Herbison, 2016). GnRH neurons exhibit pulsatile secretion and synchronized burst activities even in immortalized cells, yet the origin of the pulse generator still remains elusive (Wetsel et al., 1992).

Recent study demonstrated that pulsatile kisspeptin administration to the POA evokes synchronous GnRH promoter activity, indicating that kisspeptin inputs to GnRH neurons are important in GnRH pulse generation (Choe et al., 2013). A series of elegant studies on the driver of pulsatile GnRH activity was performed. For instance, optogenetic activation of GnRH neurons (Campos and Herbison, 2014) or the ARN kisspeptin neurons (Han et al., 2015) generated pulsatile LH secretion in both cases. In adult mice, ARN kisspeptin neurons exhibited synchronized calcium oscillations *in vivo* that correlated with pulsatile LH secretion (Clarkson et al., 2017). Kisspeptin neurons in the ARN are, thus, considered as an important regulator of the GnRH pulse generator (Herbison, 2018; McCartney and Marshall, 2014).

Kisspeptin neurons in the ARN, located on both sides of the third ventricle, develop at embryonic day 16.5 and become synaptically connected to GnRH neurons (Kumar et al., 2015), unlike another kisspeptin population in the AVPV detected at later developmental stages (Clarkson and Herbison, 2006). Kisspeptin neurons are thought to “time” reproduction by coordinating inputs for maturation and regulation of the GnRH pulse generator (Putteeraj et al., 2016). Loss-of-function mutations in Kiss1R cause the absence of sexual maturation (Seminara et al., 2003). In addition, ARN kisspeptin neurons co-expressing NKB and Dyn, known as KNDy neurons, can auto-regulate ARN kisspeptin neurons to influence GnRH neurons (Millar, 2014; Moore et al., 2018), suggesting that ARN kisspeptin neurons have a potential as the driver of the GnRH pulse generator. Indeed, LH secretion, a surrogate

marker of GnRH secretion, was tightly correlated with the calcium dynamics of ARN kisspeptin neurons (Clarkson et al., 2017). However, whether ARN kisspeptin neurons themselves can generate the rhythm has yet to be determined. Considering the finding that ARN kisspeptin neurons have been shown to coordinate circadian input from the suprachiasmatic nucleus (SCN) and control feeding, locomotor activity, body temperature, and sleep pattern (Padilla et al., 2019), it is therefore essential to address whether ARN kisspeptin neurons are able to generate rhythm on their own or simply relay neural information, and identify the inputs that are involved in their regulation.

Monitoring changes in intracellular calcium concentration using genetically encoded calcium indicators offers access to cell type-specific calcium dynamics to characterize neuronal physiology (Grienberger and Konnerth, 2012). GnRH neurons in cell culture or acute brain slices display spontaneous calcium transients that correlate with their burst firing pattern (Jasoni et al., 2010; Terasawa et al., 1999). Recently, ultradian rhythms of calcium oscillations were observed in hypothalamic slices in the SPZ-PVN that were superimposed to the circadian rhythm observed in the SCN (Wu et al., 2018). Organotypic cultures, as demonstrated in the study with SPZ-PVN, can preserve functional features of the original tissue, allowing molecular and cellular investigation in vitro (Humpel, 2015). In this study, using a single cell imaging system to elucidate the rhythm in ARN kisspeptin neurons *ex vivo*, we examined the ultradian calcium oscillation in ARN kisspeptin neurons and the regulatory components contributing to oscillation generation.

MATERIALS AND METHODS

Animals. Kiss1-IRES-Cre knock-in mice were generously provided by Dr. U. Boehm (Saarland University, Germany) (Mayer et al., 2010). Pups were housed under a 12:12 hr light-dark cycle (lights on at 7:00 a.m.) and constant temperature (22-23 °C) with their mothers before being sacrificed. Food and water were provided ad libitum. In order to identify kisspeptin neurons, Kiss1-IRES-Cre mice were crossed with Ai14, a Cre-dependent tdTomato reporter knock-in mice, to label kisspeptin neurons with tdTomato (Madisen et al., 2010). Animals were genotyped using PCR from genomic DNA of toe clips prepared by KAPA mouse genotyping kit (Roche, Basel, Switzerland) and heterozygous mice were used. GnRHp-dsLuc transgenic mice were used for angled slice culture and monitoring GnRH promoter activity (Choe et al., 2013). Animal experiments were conducted in compliance with the rules and regulations established by the Institutional Animal Care and Use Committee (IACUC) of Daegu Gyeongbuk Institute of Science and Technology (DGIST).

Organotypic slice culture. Organotypic slice culture was prepared as described previously (Choe et al., 2013), with some modifications. Briefly, the brains of the neonatal mice (postnatal day 6–8) were obtained and rapidly placed in ice-cold Gey's balanced salt solution supplemented with 10 mM HEPES (pH 7.4) and 30 mM glucose, bubbled with 5% CO_2 and 95% O_2. Coronal or horizontal slices (400 μm thickness) were prepared using a

vibratome (Leica, Nussloch, Germany). An angled slice (about 18°, 300 μm thickness) was used to include kisspeptin inputs to GnRH neurons (Liu et al., 2011) in a co-culture within 2–3 slices. The ARN region located at 4.95–5.31 mm (Paxinos et al., 2007) was collected and dissected about 1 mm long and 1 mm wide along the third ventricle. Slices were explanted onto a culture membrane (Millicell-CM; Merck Millipore, Darmstadt, Germany) in slice culture media (50% minimum essential medium, 25% Gey's balanced salt solution, 25% horse serum, 36 mM glucose, and 100 U/mL antibiotic-antimycotic). The medium was replaced every 3 days before experiments.

AAV transduction of ARN slices. Organotypic slices were transduced by adding adeno-associated virus (AAV) after the slices were stabilized (Brancaccio et al., 2013). AAV2/1-hSyn-Flex-GCaMP6m (Addgene 100838) or AAV2/1-hSyn-Flex-jRGECO1a (red-fluorescent genetically encoded calcium indicator for optical imaging, Addgene 100853) was dropped directly onto the surface of the ARN slice (1 μL per slice). After transduction, the slice was incubated for about 10 days before imaging. For pan-neuronal calcium imaging, AAV2/1-hSyn-GCaMP6m (Addgene 100841) was used to transduce non-specific neuronal population of the ARN. For chemogenetic studies, AAV encoding either hM4D(Gi)-mCherry (Addgene 44362) or hM3Dq(Gq)-mCherry (Addgene 44361) under the control of human synapsin promoter in a *Cre* DNA recombinase-dependent manner for inhibition or activation, respectively, was added after the application with Syn-Flex-GCaMP6m. The

DREADD systems were activated by clozapine-N-oxide (CNO) application (Armbruster et al., 2007). The viral titer was 2.69–3.842 e13 GC/mL (Addgene, Cambridge, USA) and was diluted in PBS to 5.38 e12–1.92 e13 GC/mL before use.

Real-time fluorescence and bioluminescence imaging. Intracellular calcium levels in ARN slices were monitored using a custom device, Circadian 700B (Live Cell Instrument, Seoul, Korea). The imaging system was composed of an EM-CCD camera (512 × 512 pixels, iXon3; Andor Technology, Belfast, UK), Nikon S Fluor 4× or 20× objective lenses, and LED-Excitation System (Live Cell Instrument) with 480 nm and 525 nm light sources used for excitation of GCaMP and RGECO, respectively. The slices were maintained in an incubating unit with the temperature controller set to 37 °C and gas mixer set to 5% CO2 (Live Cell Instrument). Fluorescence imaging was performed with an exposure time between 80 and 200 msec. Imaging without an interval and 2–3 sec intervals gave similar results, so the experiments were performed with 2–3 sec intervals with the NIS Software (Nikon, Tokyo, Japan). The fluorescence intensity of individual neurons was measured from regions of interest established for single neurons with the NIS software. Background fluorescence from each image was also measured and subtracted. Relative fluorescence changes were calculated as $\Delta F/F = (F - F0)/F0 \times 100$, where F0 was the baseline fluorescence intensity obtained by the mean fluorescence intensity from 30 sec between the peaks (average of three

values). Luminescence imaging was performed with the same device, with 1.5 min exposure time. The median value was used to filter out noise.

Drug treatments. Drugs were diluted in the recording media (DMEM:Ham's F12 medium supplemented with N2 supplement, 36 mM glucose, and 100 U/mL antibiotic-antimycotic) to the indicated concentrations and perifused at a flow rate of 2.4 mL/h. For luminescence imaging, 1.5 mM luciferin was added to the recording media. After the baseline oscillation was obtained, drug-containing media was applied for 15–30 min, and were washed out with recording media for the same time period. Tetrodotoxin (voltage-gated sodium channel blocker), 4-aminopyridine (voltage-gated potassium channel blocker), senktide (neurokinin 3 receptor [NK3R] agonist), D-AP5 (NMDAR antagonist), and bicuculline (GABAA antagonist) were purchased from Tocris (Bristol, UK). Verapamil (voltage-gated calcium channel blocker) was purchased from Sigma-Aldrich (St. Louis, USA). CNO was purchased from Enzo Life Sciences (Farmingdale, USA), dynorphin was purchased from Phoenix Pharmaceuticals (Burlingame, USA). Kisspeptin-10 (Kotani et al., 2001) and Kp-271 (Pineda et al., 2010) were synthesized from Anygen (Gwangju, Korea).

Immunohistochemistry. Kisspeptin antiserum AC566 was generously provided by Dr. M. Beltramo and Dr. A. Caraty (INRA, France) (Franceschini et al., 2006). For immunostaining, mice were perfused with 4%

paraformaldehyde (PFA) in PBS, and brains were post-fixed for 12 hr in 4% PFA before sectioning (40 μm thickness). Brain sections were collected in 12 wells, and sections in every three wells were used. Brain sections containing ARN (Paxinos et al., 2007) were blocked with 10% goat serum, 0.3% Triton X-100 in PBS, and then kisspeptin antiserum (1:2500) was applied overnight at 4 °C. For organotypic slice immunostaining, slices on culture membrane were fixed with 4% PFA, blocked with 10% goat serum, 0.5% Triton X-100 in PBS, and incubated overnight with kisspeptin antiserum at 4 °C.

Confocal microscopy. For observing the neuronal cell bodies at different depth within the slice, confocal laser scanning microscope LSM 700 (Carl Zeiss, Oberkochen, Germany) was used. The images were obtained using Z-stack imaging, by scanning from the lowest to the highest position of the fluorescence signal. For identifying kisspeptin-positive neurons among tdTomato positive neurons, LSM 800 (Carl Zeiss) was used. NeuroTrace (Invitrogen, Waltham, USA), a fluorescent stain for Nissl bodies, was used to identify positive signals within single neurons and avoid multiple counting. Cells were counted from 6–9 slices from each mouse.

Statistical Analysis. Data are presented as the mean ± standard error. Data analysis for period and amplitude of calcium oscillation was performed using the fast Fourier transformation nonlinear least squares function from the online BioDare (https://biodare2.ed.ac.uk) with adjusted time frame, and cells

that were out of the linear min-max range were excluded. Peaks were identified using Cluster analysis (Veldhuis and Johnson, 1986). Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, USA). Comparison of mean amplitude and period of kisspeptin and pan-neuronal population were performed using unpaired two-tailed Student's t-tests. Among pre-, treat, and post- (washout) phases, differences in mean amplitude and period of pre- and post-treatment were compared using paired two-tailed Student's t-test.

RESULTS

Kisspeptin neuron-specific synchronized calcium oscillation in mouse hypothalamic ARN *ex vivo*

First, I examined whether ARN kisspeptin neurons exhibit oscillations when maintained in isolation. To monitor kisspeptin neuron-specific intracellular calcium levels *ex vivo*, organotypic slice cultures of the hypothalamic ARN region obtained from neonatal *Kiss1*-IRES-Cre mice were prepared. After stabilization for approximately 10 days, kisspeptin neurons in the ARN were transduced with AAV encoding the calcium indicator in a *Cre* DNA recombinase-dependent manner (Fig. 5). To monitor single-cell intracellular calcium dynamics, fluorescence signals were acquired with a custom real-time imaging system that infuses culture media and incubates the *ex vivo* slice (Fig. 5). Before monitoring calcium oscillation, I examined the specificity of *Kiss1*-IRES-Cre driver line using immunohistochemistry. In *Kiss1*-IRES-Cre/tdTomato mice, 84.8 ± 1.92% of tdTomato-positive neurons were kisspeptin-positive (Fig. 6), suggesting that Cre-mediated expression of tdTomato in ARN of *Kiss1*-IRES-Cre mice was indeed kisspeptin-expressing neurons.

Then, I monitored calcium dynamics from kisspeptin neurons in the *ex vivo* slices as explained in Fig. 5. ARN kisspeptin neurons displayed bright

fluorescence as shown in the representative time-lapse images (Fig. 7A). Line scan imaging of the indicated vertical line revealed a robust fluctuation of the cytosolic calcium concentration of kisspeptin neurons (Fig. 7B) As a whole, the entire oscillating kisspeptin neurons revealed synchronized calcium oscillation (Fig. 7C). Each line represents a single cell. The dotted box in Fig. 7C shows that the oscillatory period is similar among kisspeptin neurons, with fluorescence intensity rising and falling collectively at a period of about 3 min with amplitude variations (Fig. 7D). Individual profiles also exhibited calcium oscillation pattern with similar period and different amplitudes throughout the time-lapse imaging (Fig. 8).

Confocal imaging of the *ex vivo* slices expressing the calcium indicator revealed differences in fluorescence intensity according to the depth of imaging positions of Z1 and Z2 (Fig. 9A). The cross-section in Fig. 9A depicts that the soma of cell #2 is located at the lower end of the slice, compared to cell #1 (Fig. 9A). The difference in the Z-axis location of cell bodies, therefore, could have contributed to the amplitude variation of the calcium intensity of kisspeptin neurons, in addition to varying kinetics (Song et al., 2012).

Synchronized calcium oscillation is specific to kisspeptin neurons in the ARN

To address whether synchronized calcium oscillation is specific to

kisspeptin neurons, I observed calcium dynamics of pan-neuronal population expressing GCaMP from organotypic slice cultures, without a neuronal type-specificity (Fig. 10A). Pan-neuronal calcium levels mainly showed irregular or an occasionally simultaneous peak with a rare chance of synchronization (Fig. 10, 11A). The non-synchronous peaks in the pan-neuronal population were not due to lack of responsiveness, as calcium signals could be evoked with depolarizing concentration of potassium chloride solution (Fig. 11B). Compared to ARN kisspeptin neuron-specific calcium oscillation, pan-neuronal calcium oscillation exhibited significantly lower amplitudes (Fig. 12) (350.7 ± 95.07 a.u. for kisspeptin, and 85.16 ± 6.99 a.u. for pan-neuronal population). The period of ARN kisspeptin neuronal calcium oscillation was precise with a homogeneous period of 3.05 ± 0.04 min compared to the irregular and heterogeneous pan-neuronal population (41.32 ± 3.86 min). The number of peaks in the same time period was significantly more in kisspeptin neurons (10.05 ± 0.09 for kisspeptin and 4.94 ± 0.20 for pan-neuronal), altogether demonstrating synchronized ultradian calcium rhythmicity specifically observed among ARN kisspeptin neurons (Fig. 12).

The calcium transients detected from two fluorescent calcium indicators, GCaMP6m and jRGECO1a, expressed from the same neuron exhibited similar oscillating patterns (Fig. 13A). There was a significant ($p < 0.001$) amplitude difference between the two calcium indicators (59.95 ± 3.20 a.u. for GCaMP and 40.58 ± 1.78 a.u. for RGECO); however, the period was not different (Fig. 13B, C). Therefore, either GCaMP or RGECO was used to

monitor calcium oscillations throughout the experiments. Then I addressed whether the preparation of *ex vivo* slice cultures may affect spontaneous synchronicity of ARN kisspeptin population. Similar to coronal slices (Fig. 7), synchronized calcium oscillation was also detected from horizontal slices (Fig. 9B), indicating that the oscillation is exhibited regardless of plane orientation. Coronal slices were used in subsequent experiments. Immunohistochemistry of organotypic slices with kisspeptin antiserum revealed anti-kisspeptin puncta in AAV-transduced ARN neurons (Fig. 9C), verifying the expression of calcium indicator specifically in kisspeptin neurons. Then, we examined whether kisspeptin neurons in AVPV exhibit calcium oscillation. However, no RGECO positive calcium signals were detected from AVPV organotypic slice culture derived from Kiss1-IRES-Cre mice transduced with $AAV_{2/1}$-hSyn-Flex-jRGECO1a (Fig. 14A). To address whether the absence of signal is due to lack of developmental onset of *Kiss1* expression, I examined the expression histologically with fluorescent reporter in Kiss1-IRES-Cre/tdTomato mice. AVPV kisspeptin neurons were not present around postnatal day 7, while at the same age, the ARN kisspeptin neuronal population is already present (Fig. 14B). Because GnRH pulsatility is observed soon after birth (Herbison, 2016), I decided to use the arcuate kisspeptin neurons in neonates to elucidate the pulsatile rhythm. These results validate that the synchronized calcium oscillation was observed specifically from ARN kisspeptin neurons compared to pan-neuronal or AVPV kisspeptin neurons, regardless of types of calcium indicators or plane orientation.

Voltage-gated ion channels involved in synchronized calcium oscillation in ARN kisspeptin neurons

To characterize whether voltage-gated ion channels are involved in operating the synchronized calcium oscillation of ARN kisspeptin neurons, voltage-gated ion channel blockers were applied to *ex vivo* ARN slices. Tetrodotoxin (TTX) is a widely used neurotoxin that binds to voltage-gated sodium channels, inhibits action potential, and blocks neuronal transmission without changing the resting potential (Nieto et al., 2012). The bath application of TTX (0.5 μM) almost completely abolished the calcium oscillations in kisspeptin neurons compared to vehicle (0.02% sodium acetate in recording media) treatment, indicating that action potential-mediated synaptic transmission is involved in the synchronized rhythm generation (Fig. 15). The calcium oscillation began to diminish a couple of peaks following TTX application, suggesting that remaining oscillating mechanism could have driven calcium oscillation prior to the elimination of synaptic input (Fig.15B, right). Kisspeptin neurons appeared sensitive to TTX because the calcium oscillation did not fully recover to the pre-treatment phase, although the oscillation started to reappear. The recovery from TTX required further incubation after the termination of TTX treatment, and the frequencies of calcium oscillation were short with slow increments of amplitude (Fig. 15B right).

Next, I applied 4-aminopyridine (4-AP), a voltage-gated potassium channel blocker, known to affect action potential duration (Strupp et al., 2017). When 4-AP (0.5 mM) was applied to the ARN, kisspeptin neuron-specific calcium oscillation was sustained at the peak level without oscillation, and gradually returned to baseline to recover the spontaneous oscillation (Fig. 16 right). Notice that the oscillatory period after 4-AP treatment was lengthened (Fig. 16B right) compared to vehicle (0.1% distilled water in recording media, Fig. 16B left). L-type voltage-gated calcium channels are important in hormone secretion and are involved in GnRH neuron calcium transients (Constantin et al., 2012; Simms and Zamponi, 2014). When L-type voltage-gated calcium channels were blocked by verapamil (VP), there was no change in synchronized calcium oscillation (Fig. 17). Taken together, these results suggest that action potential-mediated synaptic transmission is critical to generate synchronized calcium oscillation in ARN kisspeptin neurons.

Chemogenetic modulation of ARN kisspeptin neurons alters synchronized calcium oscillation

To explore whether ARN kisspeptin neuronal calcium oscillation is synchronized through the neurotransmission within the population, kisspeptin neuron-specific synapses were manipulated using the DREADDs (designer receptors exclusively activated by designer drugs) system (Roth, 2016; Stachniak et al., 2014). For kisspeptin neuron-specific inhibition, ARN slices

were sequentially transduced with AAV encoding GCaMP6m and another AAV encoding hM4Di-mCherry in a kisspeptin neuron-specific manner (Fig. 18A). ARN kisspeptin neurons showed positive signals for both GCaMP and hM4Di-mCherry after transduction (Fig. 19A). The percentage GCaMP6m+, hM4Di-mCherry+ or GCaMP6m+/hM4Di-mCherry+ double positive kisspeptin neurons are shown in Fig. 20B and the proportions of double positive neurons in GCaMP6m+ or hM4Di-mCherry+ population are shown in the right (Fig. 19B). Among GCaMP6m-positive kisspeptin neurons, 24.01 ± 4.17% were positive for mCherry, while 63.13 ± 11.23% of hM4Di-mCherry-positive neurons were positive for GCaMP6m (Fig. 19B). When the DREADD ligand clozapine-N-oxide (CNO, 1 μM) was applied to ARN kisspeptin neurons, calcium oscillation was dampened (Fig. 18C) while vehicle (0.01% distilled water in recording media) treatment did not show any difference (Fig. 18B). About one-fourth of GCaMP-positive ARN kisspeptin neurons were mCherry-positive, yet the synchronized calcium oscillation in all GCaMP-positive kisspeptin neurons was inhibited by CNO (Fig. 18C), indicating that transmission from hM4Di-positive kisspeptin neurons can inhibit the rest. Nevertheless, calcium oscillation resumed early even before washout (Fig. 18C), suggesting that transmission from hM4Di-negative kisspeptin neurons was sufficient to restore the synchronized calcium oscillation. The period of oscillation significantly ($p < 0.001$) increased after CNO-induced inhibition, without change of amplitude (Fig. 18D).

For kisspeptin neuron-specific activation, hM3Dq-mCherry was

expressed in the similar scheme with the chemogenetic inhibition (Fig. 19A). When CNO was applied to ARN slices, the baseline of kisspeptin neuron-specific calcium oscillation increased compared to pre-treatment (Fig. 20A). Since kisspeptin neurons oscillate with an endogenous rhythm, not silent, I reasoned that over-activation caused the increased baseline of calcium oscillation. Interestingly, the amplitude significantly ($p < 0.001$) decreased despite the increased baseline, while the period was similar when compared to pre- and post- treatment (Fig. 20B). The fold change of baseline increase during CNO application was quantified for individual ARN kisspeptin neurons (Fig. 20C). Among 23 neurons from one batch, the baseline of 10 neurons increased above average, whereas those of 13 neurons did not show much increase (Fig. 20C). These results suggest that neural transmission among ARN kisspeptin neurons play a crucial role in generating the synchronized calcium oscillation.

Effect of neuropeptides in KNDy neurons on synchronized calcium oscillations

ARN kisspeptin neurons are also known as KNDy neurons as they co-express the neuropeptides kisspeptin, neurokinin B, and dynorphin (DYN), possibly forming an auto-regulatory network (Moore et al., 2018). To examine whether these neuropeptides released from KNDy neurons could mediate the synchronized calcium oscillation, I treated the ARN slices with kisspeptin,

tachykinin NK3R agonist (senktide), and DYN. When ARN slices were treated with kisspeptin, calcium oscillation was temporally suppressed (Fig. 21). After washout, the period was significantly ($p < 0.001$) lengthened while the amplitude was reduced (Fig. 21). Although ARN kisspeptin neurons do not express kisspeptin receptor Kiss1R, other ARN neuronal populations expressing Kiss1R, such as proopiomelanocortin (POMC) neurons (Higo et al., 2017) may mediate the kisspeptin-elicited response. Also, based on the studies where NKB can stimulate and DYN can inhibit kisspeptin neurons (Millar, 2014; Moore et al., 2018), I examined whether signal transduction via senktide, an NK3R agonist known to activate ARN kisspeptin neurons, could induce calcium oscillation while DYN can inhibit them. Treatment of ARN slices with senktide significantly ($p < 0.001$) decreased the period and amplitude of kisspeptin neuron-specific calcium oscillation in the post-treatment session (Fig. 22). When DYN was applied to ARN slices, calcium oscillation temporally and slightly diminished (Fig. 23). In the post-treatment phase, the period of calcium oscillation slightly decreased while the amplitude remained unchanged (Fig. 23), indicating that the KNDy components are marginally involved in regulating the ARN kisspeptin neuron-specific synchronized calcium oscillation by affecting the period and amplitude.

Glutamatergic and GABAergic effects in ARN kisspeptin neuron-specific calcium oscillation

Electrophysiological properties of ARN kisspeptin neurons can be modulated by excitatory or inhibitory synaptic transmission. For example, ARN kisspeptin neurons could generate burst firing in an *N*-methyl-D-aspartate (NMDA) receptor-dependent manner (Gottsch et al., 2011), whereas $GABA_A$ receptor-mediated inhibitory inputs affected postsynaptic currents (DeFazio et al., 2014). Based on these previous studies, I examined whether NMDA receptor- or $GABA_A$ receptor-dependent inputs into ARN kisspeptin neurons can affect the synchronized calcium oscillation. The application of NMDA receptor antagonist D-AP5 (50 μM) to ARN organotypic slices immediately silenced calcium oscillation in kisspeptin neurons (Fig. 24). After washout, the suppressed calcium oscillation was released within a few minutes and the synchronized oscillation was resumed. Compared to the pre-treatment session, the period of oscillation was significantly ($p < 0.01$) lengthened in the post-treatment session, while the amplitude remained similar before and after the treatment (Fig. 24). When $GABA_A$ receptor antagonist bicuculline (BIC, 10 μM) was applied, calcium oscillation was temporally dampened (Fig. 25). Interestingly, the oscillation amplitude decreased, and the period increased significantly ($p < 0.001$) at the post-treatment phase (Fig. 25). This pattern was similar when treated for 15 min or 30 min. These data suggest that NMDA receptor-mediated synaptic transmission have a critical role, and $GABA_A$ receptor-mediated synaptic transmissions a modulatory action, in kisspeptin neuron-specific calcium oscillation.

GnRH neuron activity and the effect of kisspeptin

To address whether ARN kisspeptin neurons affect ultradian oscillations in GnRH neurons, GnRH gene expression was monitored from transgenic mice bearing a destabilized luciferase under the control of the GnRH promoter (GnRHp-dsLuc) (Choe et al., 2013). An angled slice culture was prepared to obtain brain sections containing kisspeptin neurons and GnRH neurons in a co-culture within 2 to 3 slices (Fig 26A). Luminescence imaging was performed on the same device without LED excitation and adding luciferin to the recording media (Fig. 26B). Luminescence signals were detected from scattered GnRH neurons, which is a typical distribution of POA GnRH neurons (Fig. 26C). Kisspeptin neurons were identified in the angled slice by immunostaining (Fig. 26D). When single GnRH neurons were analyzed under basal conditions, the activity of GnRHp-dsLuc appeared to be sporadically oscillating (Fig. 27), but the pattern was difficult to correlate with the robust calcium oscillation observed in ARN kisspeptin neurons (Fig. 7). To test whether GnRH neurons in the angled organotypic slice culture maintain the responsiveness, kisspeptin (Kp, 10 nM) was applied in a pulsatile manner following the protocol our group previously developed (Fig. 28) (Choe et al., 2013). GnRH neurons in the angled slice exhibited robust transcriptional responses to kisspeptin pulses, suggesting that the current preparation properly maintains the function of GnRH neurons, at least in terms of pulsatile

responsiveness. Then, I tested whether the inhibition of kisspeptinergic signaling may affect the sporadic expression of GnRH in the co-culture of kisspeptin neurons and GnRH neurons. When slices were treated with kisspeptin antagonist Kp-271 (10 μM), the sporadic oscillating pattern was indeed temporarily dampened in some, but not all, GnRH neurons (Fig. 29). Luciferase activity increased soon after it was dampened and resumed to the pre-treatment state after washout (Fig. 29). The partial inhibition may be due to a small subset of synchronized activity, as previously reported (Choe et al., 2013). These results suggest that kisspeptin neurons in the organotypic slice play a regulatory role in GnRH gene expression and imply that the activities of kisspeptin neurons *ex vivo* may be functionally relevant to the modulation of ultradian transcriptional activities of GnRH neurons.

Figure 5. Overall experimental strategy. From postnatal day 6 to 8 day of *Kiss1*-IRES-Cre mice, the hypothalamic ARN organotypic slice culture was prepared and stabilized. Then, adeno-associated virus (AAV) encoding the calcium indicator in an inverted orientation was transduced. Real-time imaging was performed with media perfusion while incubated in humidified culture chamber. Fluorescent signal was excited by LED light source and imaged by EM-CCD camera.

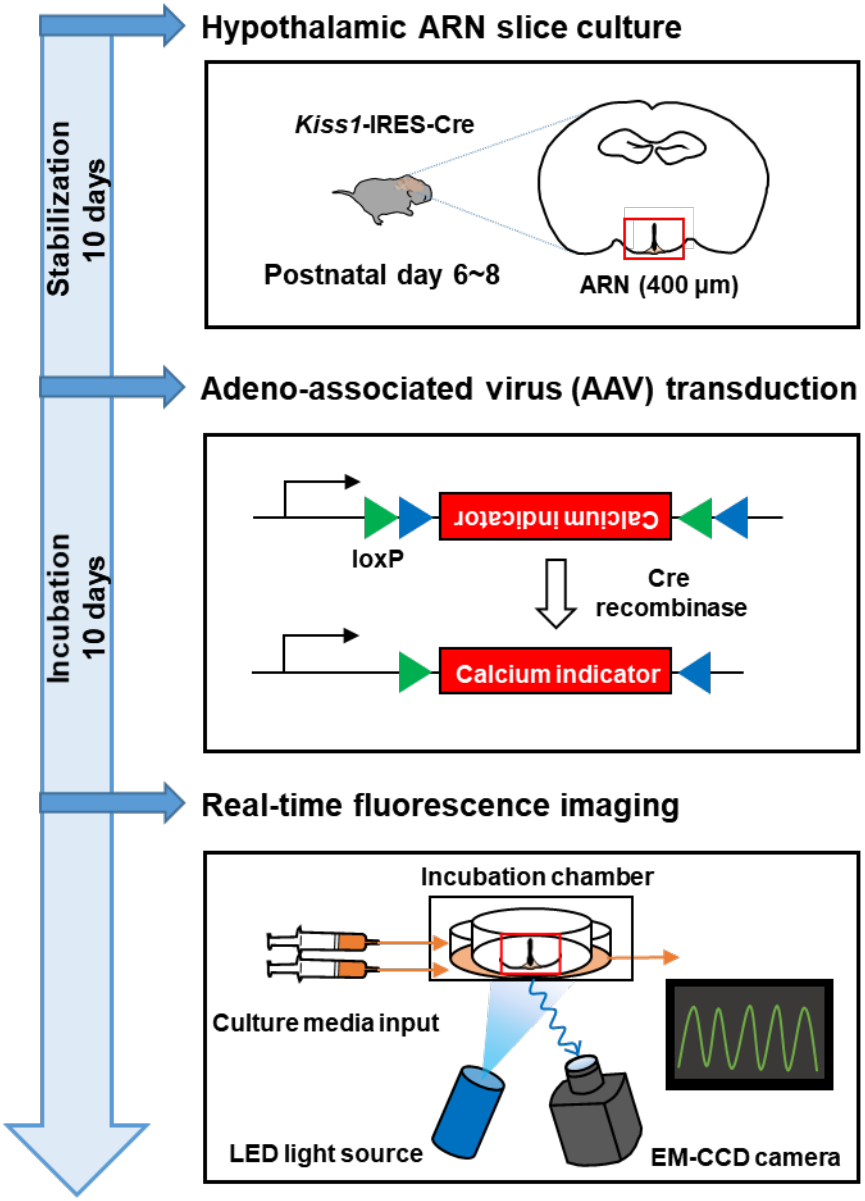

Stabilization
10 days
Incubation
10 days
Hypothalamic ARN slice culture
Kiss1-IRES-Cre
Postnatal day 6~8
ARN (400 μm)
Adeno-associated virus (AAV) transduction
Calcium indicator
loxP
Cre
recombinase
Calcium indicator
Real-time fluorescence imaging
Incubation chamber
Culture media input
LED light source
EM-CCD camera

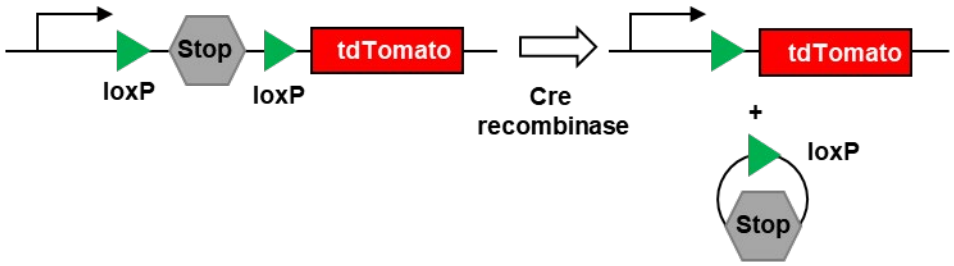
Figure 6. Kisspeptin neuron-specific signals. For the experimental model validation, *Kiss1*-IRES-Cre mice was crossed with Ai14, a Cre-dependent tdTomato expressing mice. tdTomato is expressed only in the cells that have the Cre recombinase, by the excision of a stop casette. Immunostaining of Kiss1-IRES-Cre/tdTomato mice at postnatal day 7 with kisspeptin antiserum, with 85% of Cre-expressing neurons as kisspeptin-positive (n = 14 sectons from 2 mice). Scale bar: 20 μm.

Model: Kiss1-IRES-Cre mice

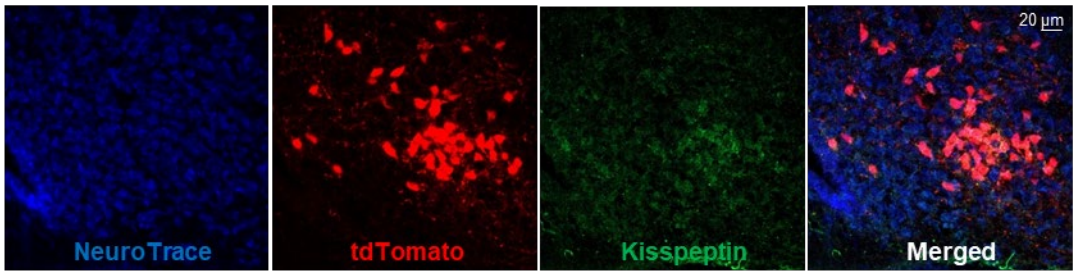
Kiss1-IRES-Cre
(2010 PNAS)

x

Ai14
(Rosa-CAG-LSL-tdTomato-WPRE)



❖ **Validation with kisspeptin antiserum**

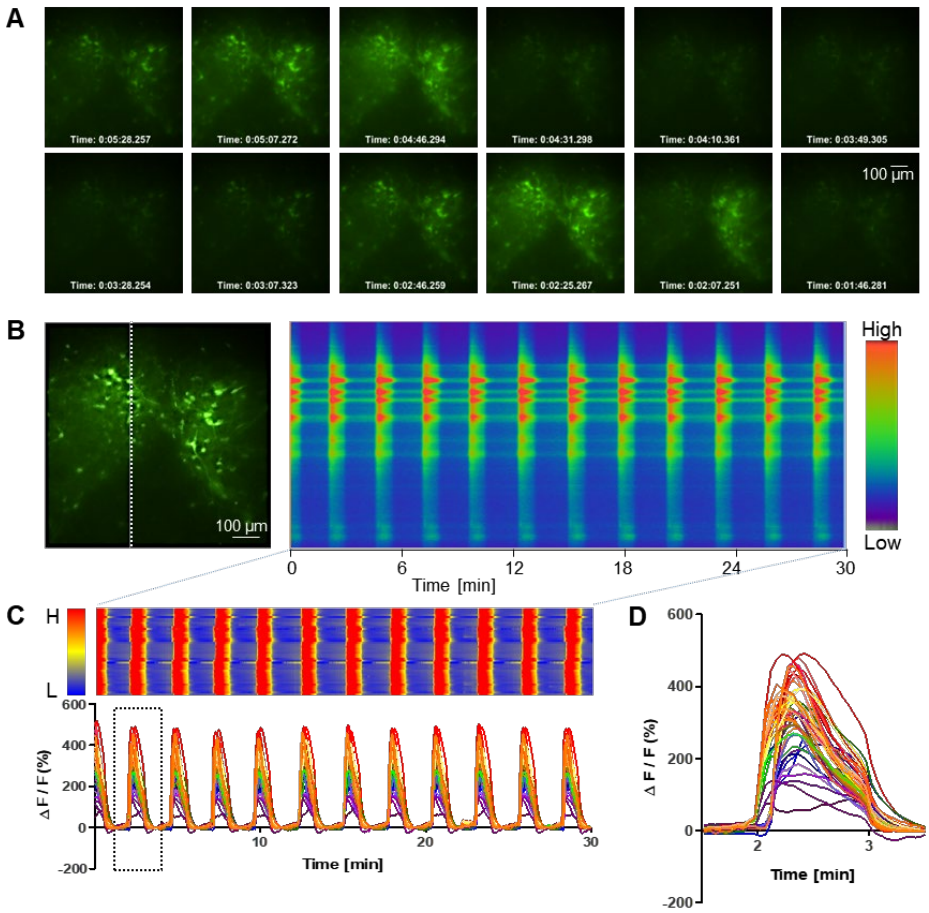


(*n* = 14 sections from 2 mice)

$$\frac{\text{Kisspeptin}^{+}\ \text{tdTomato}^{+}}{\text{tdTomato}^{+}} = 84.8 \pm 1.92\ \%$$

Figure 7. Kisspeptin neuron-specific synchronized calcium oscillation in mouse hypothalamic ARN *ex vivo*

(A) Representative time-lapse images of ARN kisspeptin neurons. Scale bar: 100 μm. (B) Change in fluorescence intensity of the calcium indicator GCaMP quantified from the time-lapse representation of the indicated vertical line. Scale bar: 100 μm. (C) Raster plot and quantified graph of calcium oscillation in time-lapse recording for 30 min (n = 44 from one batch) Each row in the raster plot represent an individual cell. (D) Single peak magnified from the dotted box in (C) for close inspection.


A
Time: 0:05:28.257
Time: 0:05:07.272
Time: 0:04:46.294
Time: 0:04:31.298
Time: 0:04:10.361
Time: 0:03:49.305
Time: 0:03:28.254
Time: 0:03:07.323
Time: 0:02:46.259
Time: 0:02:25.267
Time: 0:02:07.251
Time: 0:01:46.281
100 µm
B
100 µm
High
Low
0
6
12
18
24
30
Time [min]
C
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L
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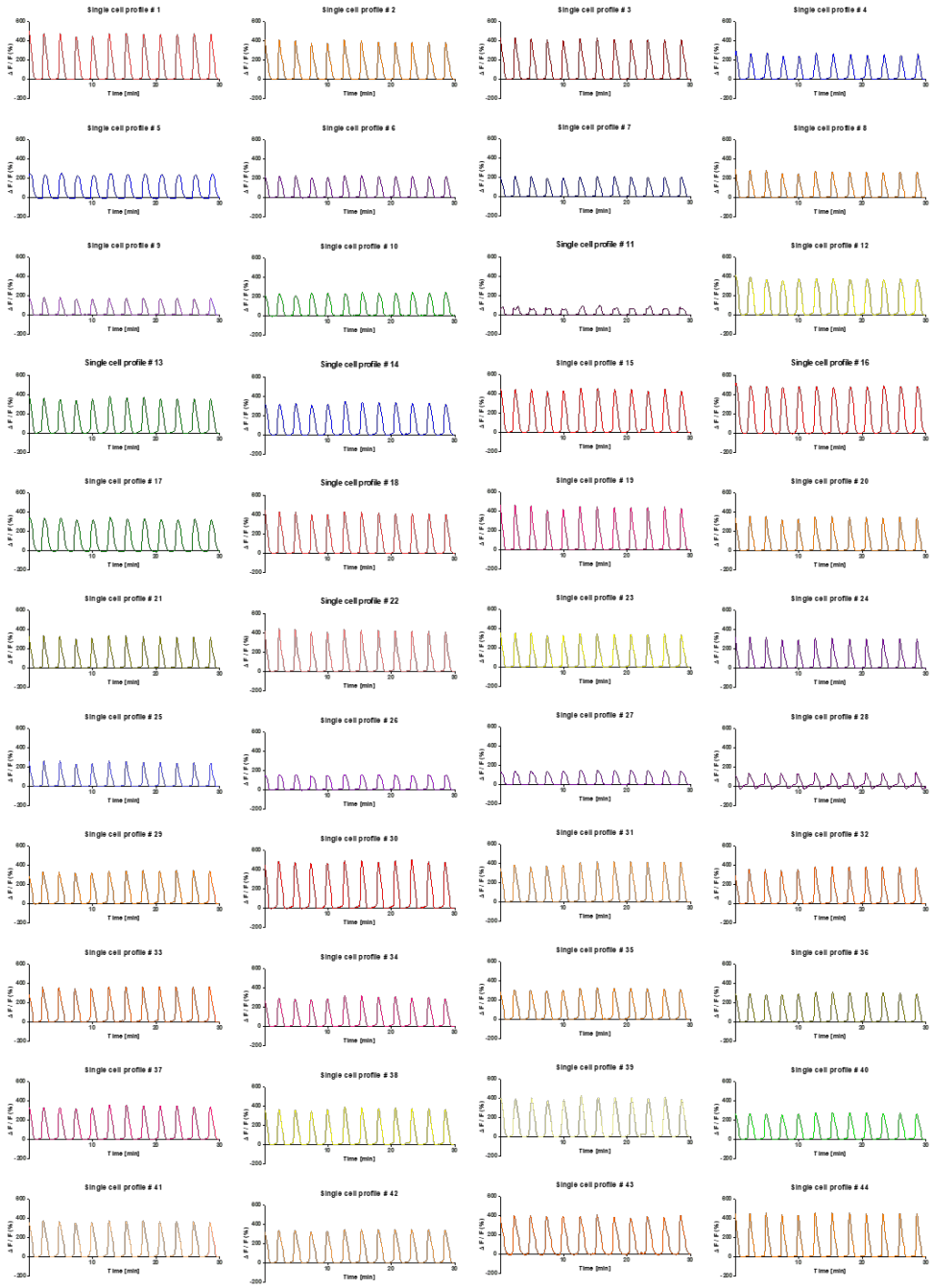
-200
Δ F / F (%)
10
20
30
Time [min]
D
600
400
200
0
-200
Δ F / F (%)
2
3
Time [min]

Figure 8. Individual profiles of kisspeptin neuron-specific calcium oscillation. Single neurons in Fig. 6 were plotted for individual calcium profile (n = 44 from one batch).


Single cell profile # 1
Single cell profile # 2
Single cell profile # 3
Single cell profile # 4
Single cell profile # 5
Single cell profile # 6
Single cell profile # 7
Single cell profile # 8
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Single cell profile # 10
Single cell profile # 11
Single cell profile # 12
Single cell profile # 13
Single cell profile # 14
Single cell profile # 15
Single cell profile # 16
Single cell profile # 17
Single cell profile # 18
Single cell profile # 19
Single cell profile # 20
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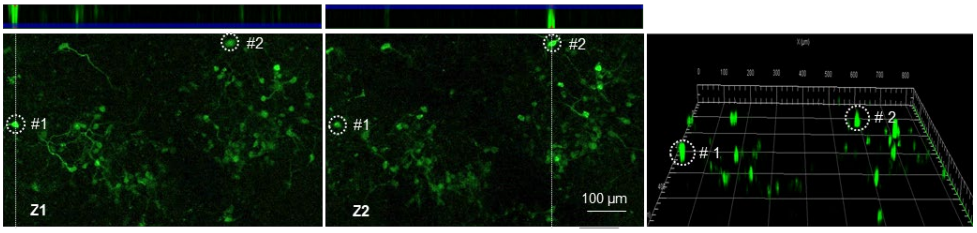
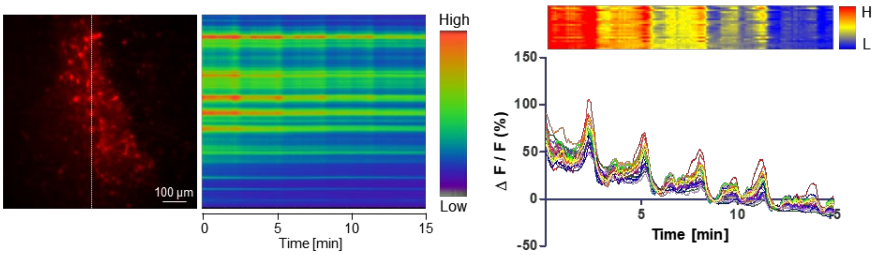
Single cell profile # 31
Single cell profile # 32
Single cell profile # 33
Single cell profile # 34
Single cell profile # 35
Single cell profile # 36
Single cell profile # 37
Single cell profile # 38
Single cell profile # 39
Single cell profile # 40
Single cell profile # 41
Single cell profile # 42
Single cell profile # 43
Single cell profile # 44
Δ F / F (%)
Time [min]

Figure 9. Validation of calcium oscillation in ARN kisspeptin neurons. (A) Confocal imaging from different depth (z-axis) of imaging of the cell body located in the organotypic slice. Cell #1 and Cell #2 are indicated in circles. 3D representation of the Z-stack image is present at right. (B) Calcium oscillation from different plane orientation. Synchronized calcium oscillation from horizontal slice kisspeptin neurons. Scale bar: 100 μm. (C) Immunostaining of RGECO-transduced neuron from organotypic slice culture with kisspeptin antiserum. RGECO-transduced neuron from the organotypic slice detected with kisspeptin antiserum. Scale bar: 5 μm

A


#2
#1
Z1
#2
#1
Z2
100 µm
2
1

B


100 µm
High
Low
0
5
10
15
Time [min]
H
L
150
100
50
0
-50
Δ F / F (%)
5
10
15
Time [min]

C

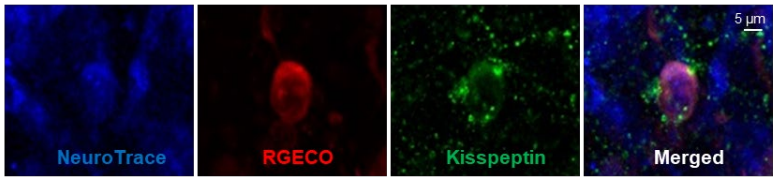

NeuroTrace
RGECO
Kisspeptin
Merged
5 µm

Figure 10. Pan-neuronal calcium oscillation. (A) Transduction of GCaMP expressing-AAV in wild type mice, without neuronal cell type-specificity. (B) Representative image and change in fluorescence intensity GCaMP from the time-lapse representation of the indicated vertical line. (C) Raster plot and changes in fluorescence intensity (n = 28 from one batch).

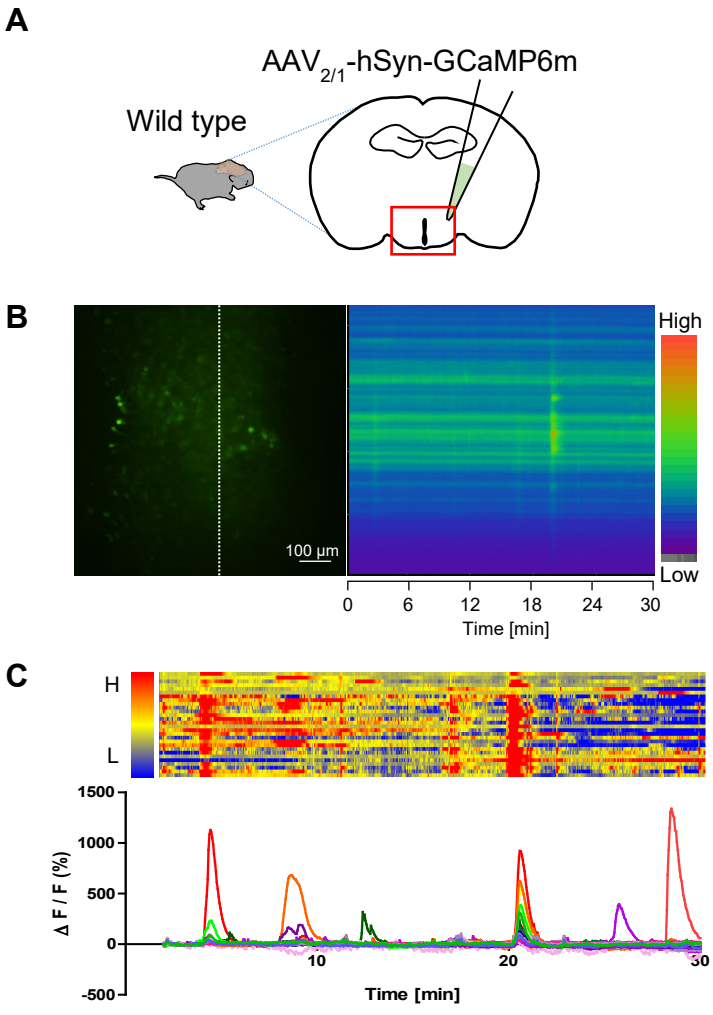

A
AAV2/1-hSyn-GCaMP6m
Wild type
B
100 μm
High
Low
0
6
12
18
24
30
Time [min]
C
H
L
1500
1000
500
0
-500
Δ F / F (%)
10
20
30
Time [min]

Figure 11. Individual profiles of pan-neuronal calcium oscillation. (A) Single neurons in Fig. 9 were plotted for individual calcium profile (n = 28 from one batch). (B) Induced calcium activity after potassium chloride (KCl, 64 mM) treatment.

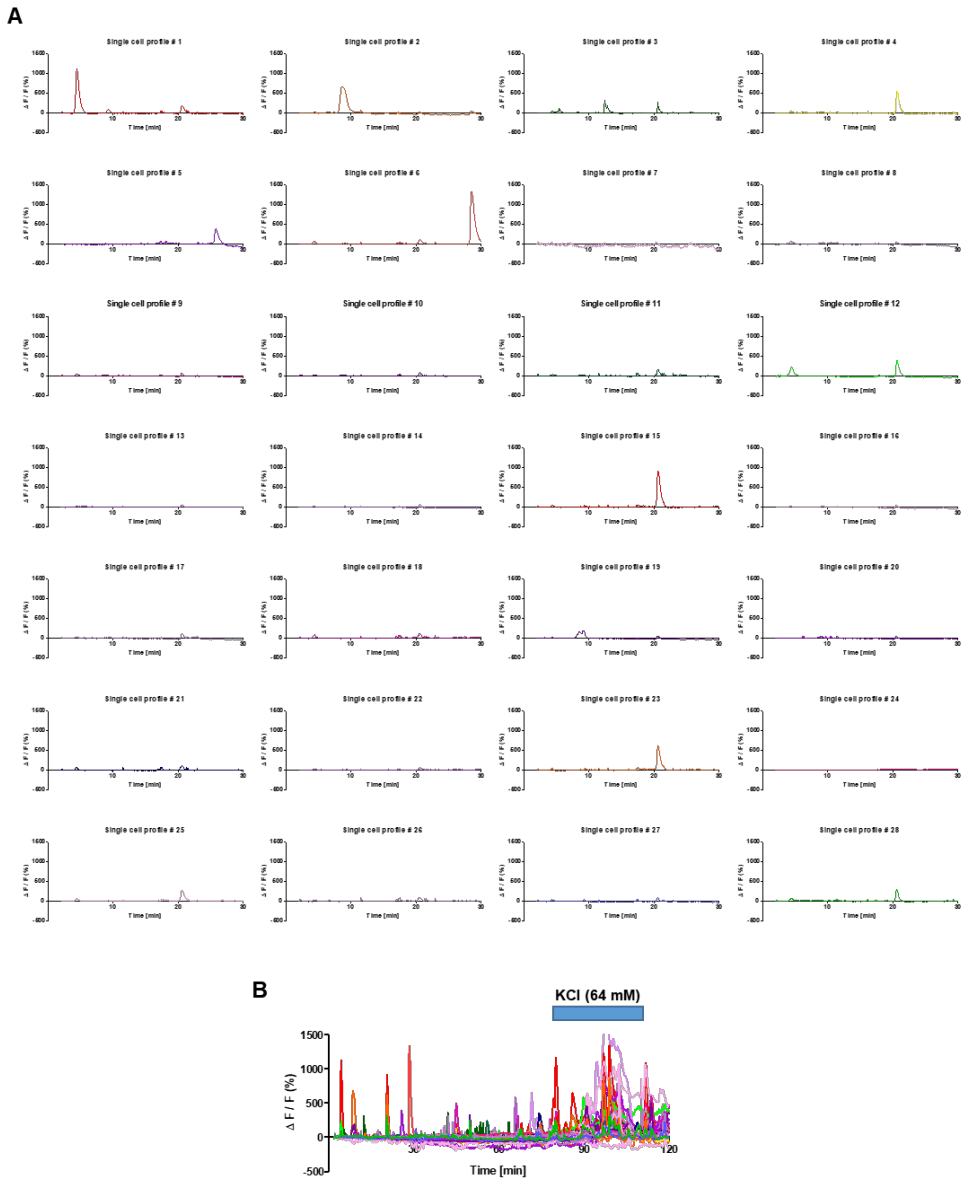
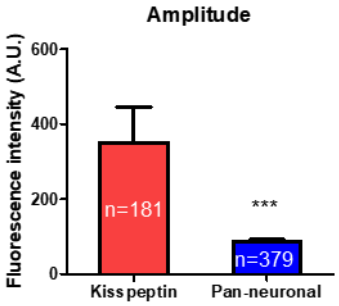
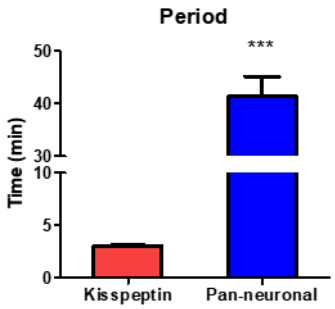

A
Single cell profile # 1
Single cell profile # 2
Single cell profile # 3
Single cell profile # 4
Single cell profile # 5
Single cell profile # 6
Single cell profile # 7
Single cell profile # 8
Single cell profile # 9
Single cell profile # 10
Single cell profile # 11
Single cell profile # 12
Single cell profile # 13
Single cell profile # 14
Single cell profile # 15
Single cell profile # 16
Single cell profile # 17
Single cell profile # 18
Single cell profile # 19
Single cell profile # 20
Single cell profile # 21
Single cell profile # 22
Single cell profile # 23
Single cell profile # 24
Single cell profile # 25
Single cell profile # 26
Single cell profile # 27
Single cell profile # 28
Time [min]
B
KCl (64 mM)
Δ F / F (%)
Time [min]

Figure 12. Statistical analysis of calcium oscillation in kisspeptin neurons and pan-neuronal population. Comparing kisspeptin and pan-neuronal calcium oscillations, the amplitude, period, and the number of peaks. (n = 181-379 from 4-5 batches, *** $p < 0.001$ by Student's t-test).


Amplitude
Fluorescence intensity (A.U.)
600
400
200
0
n=181
n=379
***
Kisspeptin
Pan-neuronal


Period
Time (min)
50
40
30
10
5
0
***
Kisspeptin
Pan-neuronal

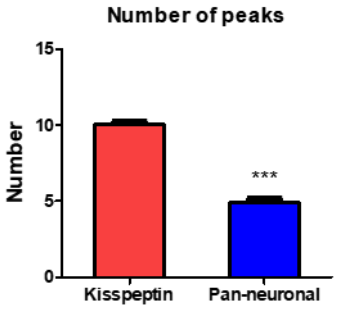

Number of peaks
Number
15
10
5
0
***
Kisspeptin
Pan-neuronal

Figure 13. Comparison of two fluorescent calcium indicators. (A) Transduction of GCaMP and RGECO in a kisspeptin neuron-specific manner. (B) Calcium transients detected from two fluorescent calcium indicators, GCaMP6m and jRGECO1a, expressed from the same neuron. (C) Amplitude and period of calcium oscillation. (n = 78 from 3 batches, *** $p < 0.001$ by Student's t-test)

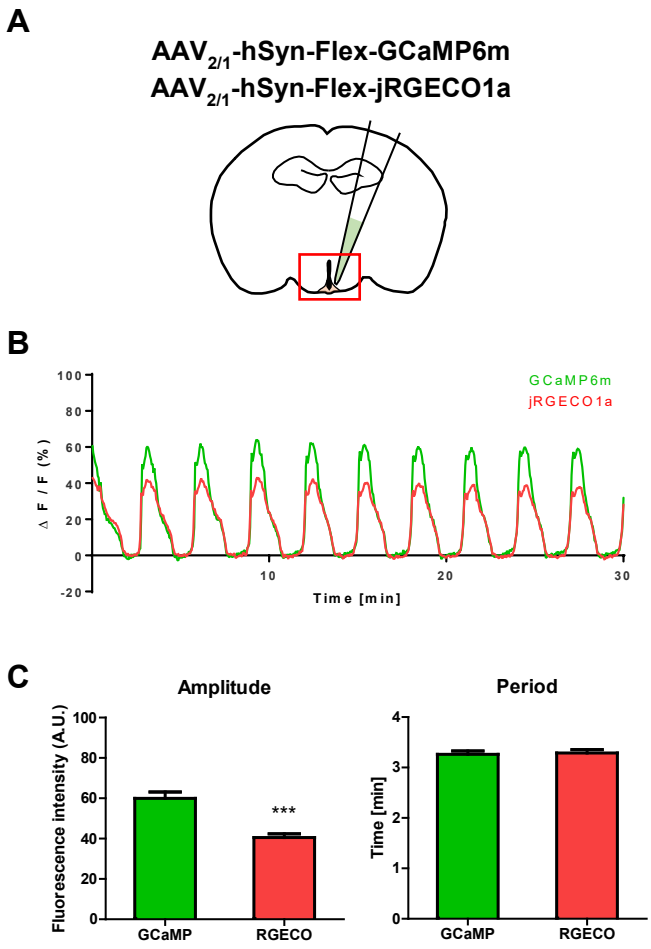

A
AAV2/1-hSyn-Flex-GCaMP6m
AAV2/1-hSyn-Flex-jRGECO1a
B
GCaMP6m
jRGECO1a
Δ F / F (%)
Time [min]
C
Amplitude
Fluorescence intensity (A.U.)
***
GCaMP
RGECO
Period
Time [min]
GCaMP
RGECO

Figure 14. Kisspeptin neurons in AVPV. (A) AVPV region transduced with RGECO. Scale bar: 200 μm. (B) tdTomato expression in AVPV and ARN kisspeptin neurons in postnatal day 7 of *Kiss1*-IRES-Cre/tdTomato mice. Scale bar: 100 μm.

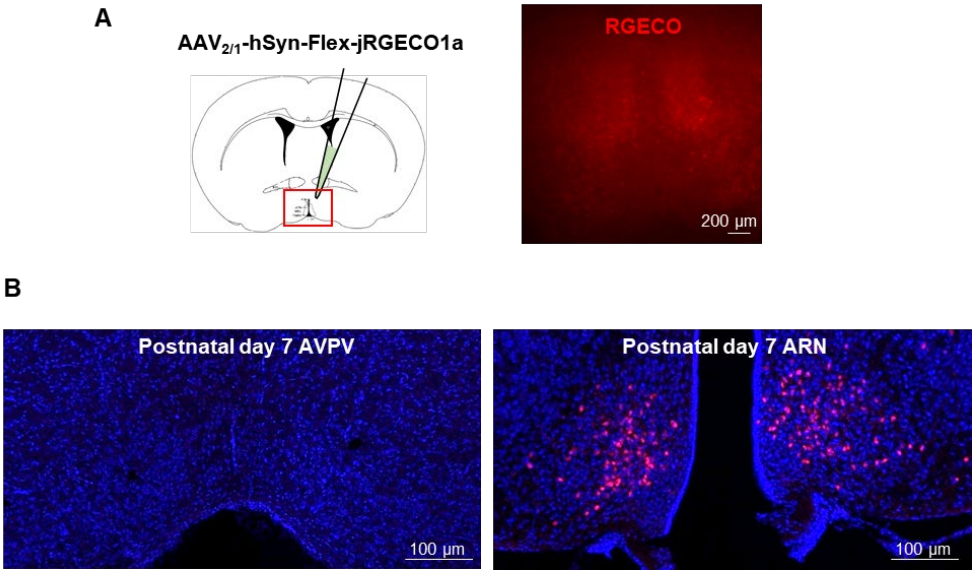

A
AAV2/1-hSyn-Flex-jRGECO1a
RGECO
200 µm
B
Postnatal day 7 AVPV
100 µm
Postnatal day 7 ARN
100 µm

Figure 15. Effect of voltage-gated sodium channel blocker in calcium oscillation in ARN kisspeptin neurons. (A) Representative image and change in fluorescence intensity of RGECO from the time-lapse representation of the indicated vertical line with voltage-dependent sodium channel blocker (TTX, 0.5 μM) application. Scale bar: 200 μm. (B) Raster plot and quantified graph of calcium oscillation with vehicle (0.02% sodium acetate in recording media) and TTX treatment.

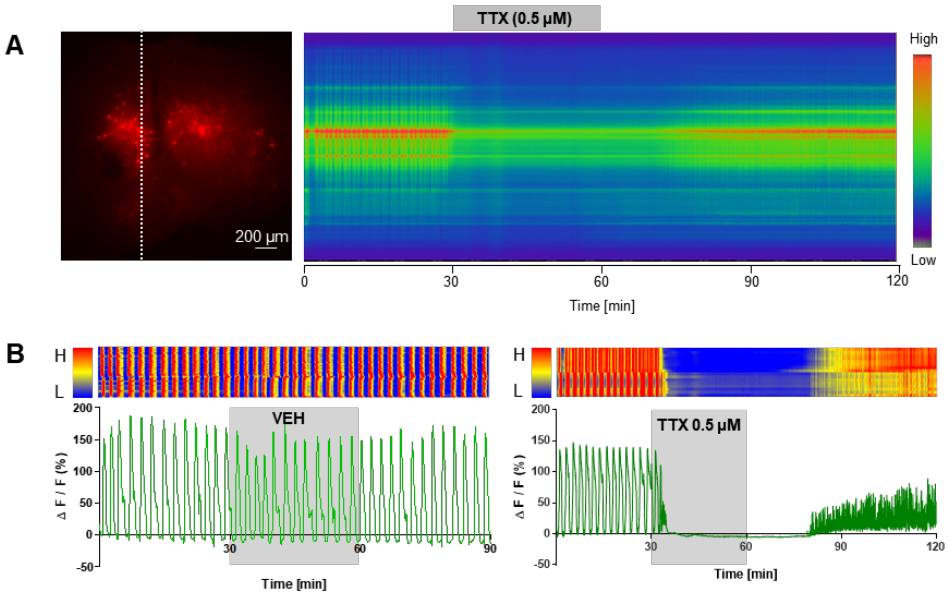

A
TTX (0.5 μM)
200 μm
High
Low
0
30
60
90
120
Time [min]
B
H
L
VEH
Δ F / F (%)
200
150
100
50
0
-50
30
60
90
Time [min]
H
L
TTX 0.5 μM
Δ F / F (%)
200
150
100
50
0
-50
30
60
90
120
Time [min]

Figure 16. Effect of voltage-gated potassium channel blocker in calcium oscillation in ARN kisspeptin neurons. (A) Representative image and change in fluorescence intensity of RGECO from the time-lapse representation of the indicated vertical line with voltage-dependent potassium channel blocker (4-AP, 0.5 mM) application. Scale bar: 100 μm. (B) Raster plot and quantified graph of calcium oscillation with vehicle (0.1% distilled water in recording media) and 4-AP treatment.

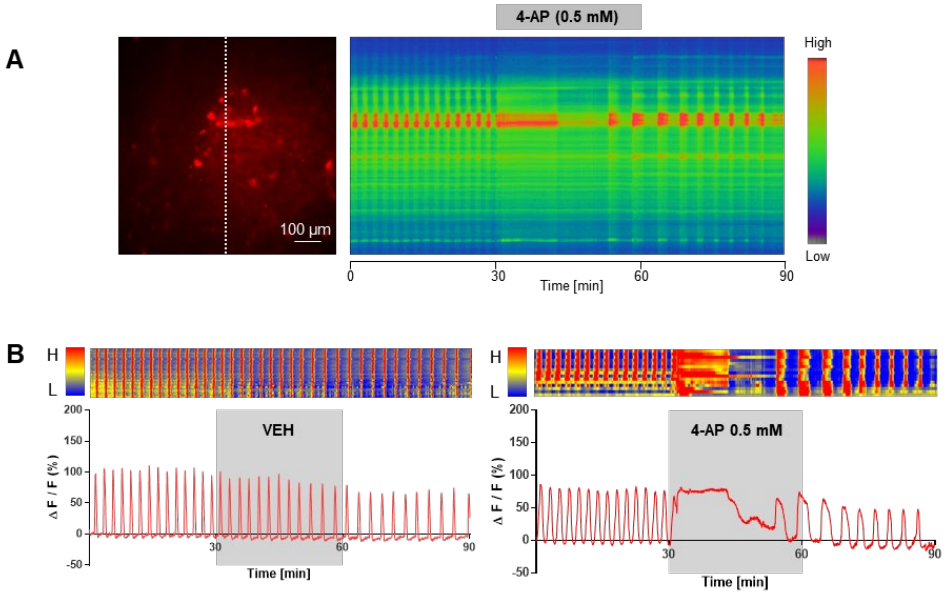

A
4-AP (0.5 mM)
100 µm
High
Low
0
30
60
90
Time [min]
B
H
L
VEH
4-AP 0.5 mM
ΔF / F (%)
200
150
100
50
0
-50
30
60
90
Time [min]

Figure 17. Effect of L-type voltage-gated calcium channel blocker in calcium oscillation in ARN kisspeptin neurons. (A) Representative image and change in fluorescence intensity of RGECO from the time-lapse representation of the indicated vertical line with L-type voltage-dependent calcium channel blocker, verapamil (VP, 50 μM) application. Scale bar: 100 μm. (B) Quantified graph of calcium oscillation with vehicle (0.03% methanol) and VP treatment.

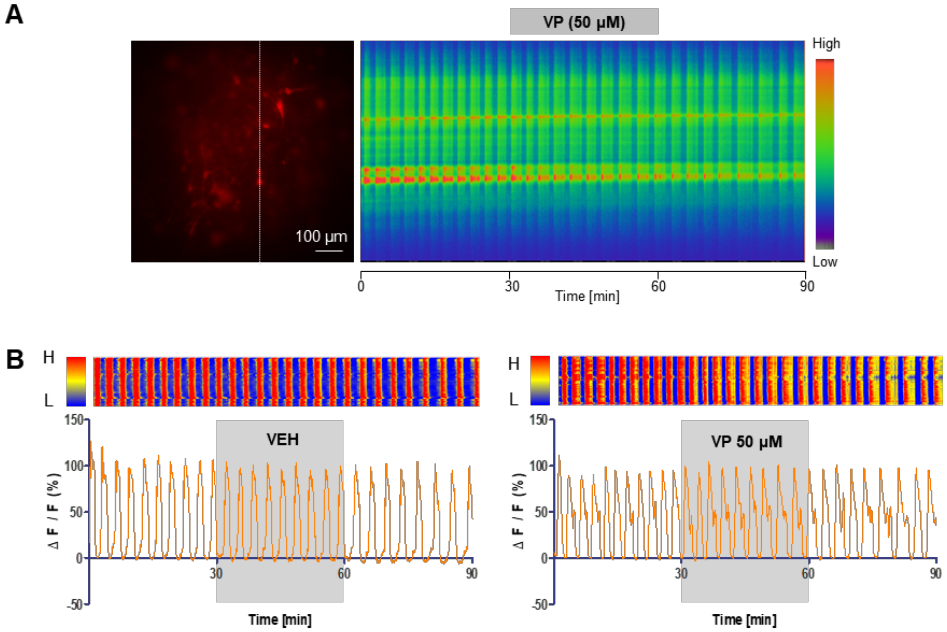

A
VP (50 µM)
High
Low
100 µm
0
30
60
90
Time [min]
B
H
L
VEH
VP 50 µM
Δ F / F (%)
150
100
50
0
-50
30
60
90
Time [min]

Figure 18. Chemogenetic inhibition of ARN kisspeptin neurons. (A) Experimental scheme for chemogenetic modulation of ARN kisspeptin neurons and calcium imaging. (B) Control (CTL, 0.01% distilled water in recording media) treatment for DREADD experiment. (C) Representative image and change in fluorescence intensity of GCaMP from the time-lapse representation of the indicated vertical line with DREADD ligand (CNO, 1 μM) application. Raster plot and quantified graph of calcium oscillation with CNO treatment in hM4Di (inhibition). (D) Amplitude and period of calcium oscillation before and after CNO treatment in hM4Di (n = 56 from 2 batches, *** $p < 0.001$ by Student's t-test).

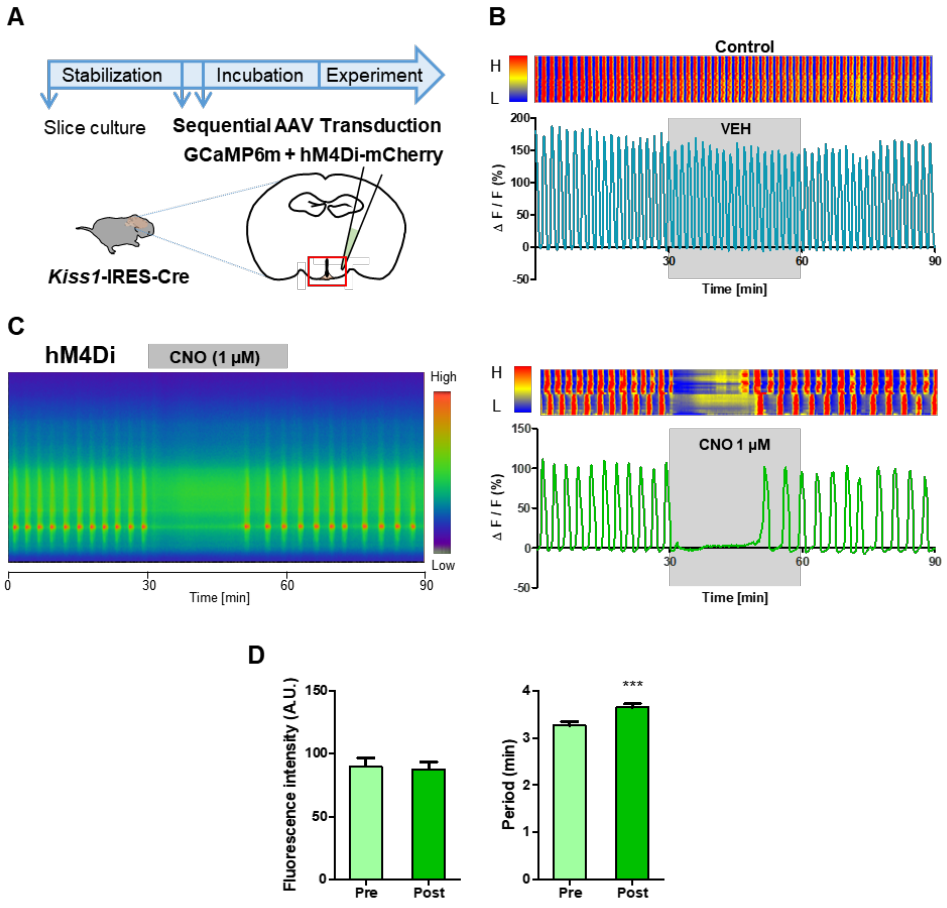
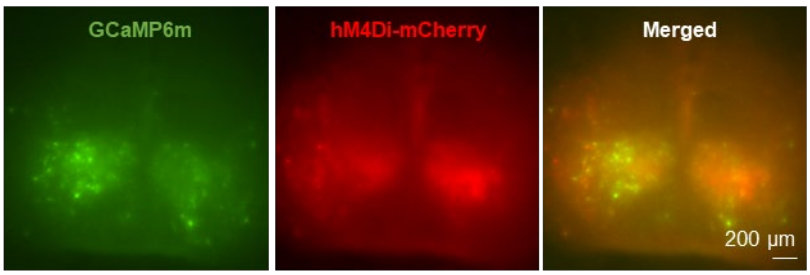

A
Stabilization
Incubation
Experiment
Slice culture
Sequential AAV Transduction
GCaMP6m + hM4Di-mCherry
Kiss1-IRES-Cre
B
Control
H
L
VEH
Δ F / F (%)
Time [min]
C
hM4Di
CNO (1 μM)
High
Low
Time [min]
CNO 1 μM
D
Fluorescence intensity (A.U.)
Pre
Post
Period (min)
***

Figure 19. Co-expression analysis of the calcium indicator and the chemogenetic system. (A) Representative image of kisspeptin neurons expressing GCaMP6m and hM4Di-mCherry, and both. Scale bar: 200 μm. (B) Percentage of kisspeptin neurons expressing GCaMP, mCherry, and both, in pie chart. Double positive neurons in respect of each GCaMP and mCherry in bar graph.

A


GCaMP6m
hM4Di-mCherry
Merged
200 µm

B

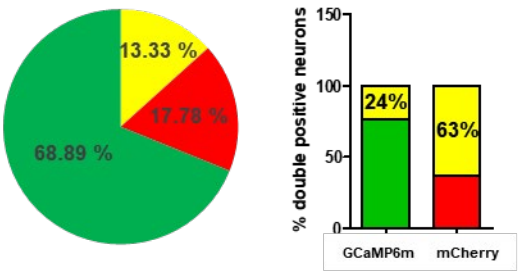

13.33 %
17.78 %
68.89 %
% double positive neurons
150
100
50
0
24%
63%
GCaMP6m
mCherry

Figure 20. Chemogenetic activation of ARN kisspeptin neurons. (A) Raster plot and quantified change in fluorescence intensify of GCaMP with CNO treatment in hM3Dq (activation). (B) Amplitude and period of calcium oscillation before and after CNO treatment in hM3Dq (n = 62 from 2 batches, *** $p < 0.001$ by Student's t-test). (C) Fold change of individual neurons transduced with hM3Dq after CNO treatment. Dotted line indicates the average value.

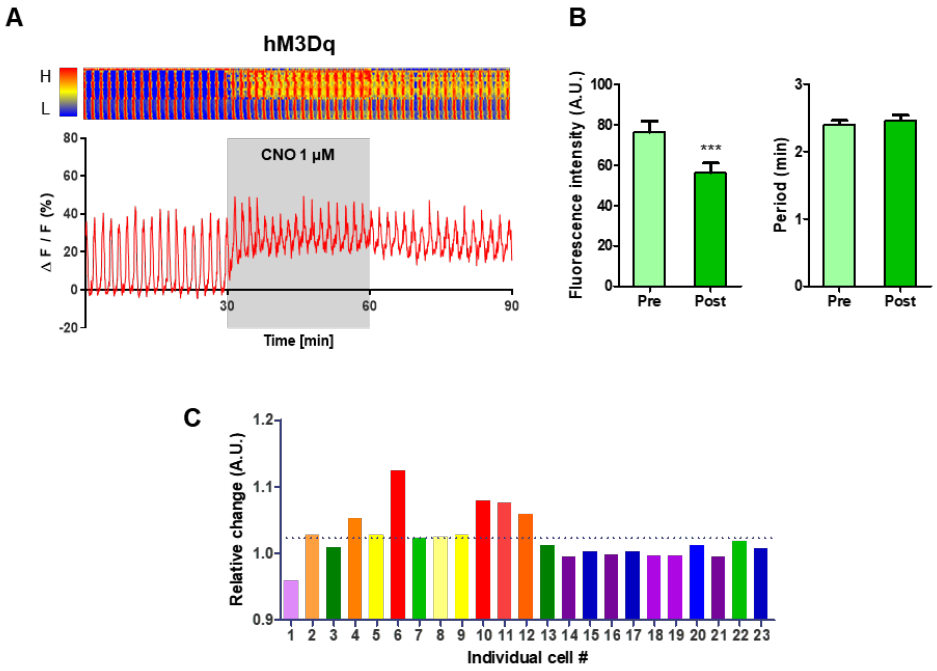

A
hM3Dq
H
L
CNO 1 µM
Δ F / F (%)
80
60
40
20
0
-20
30
60
90
Time [min]
B
Fluorescence intensity (A.U.)
100
80
60
40
20
0
***
Pre
Post
Period (min)
3
2
1
0
Pre
Post
C
Relative change (A.U.)
1.2
1.1
1.0
0.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Individual cell #

Figure 21. Effect of kisspeptin treatment on ARN kisspeptin neuron-specific calcium oscillations. (A) Representative image and change in fluorescence intensity of RGECO from the time-lapse representation of the indicated vertical line with kisspeptin (Kp, 20 nM) treatment. Scale bar: 100 μm. (B) Raster plot and quantified graph of calcium oscillation with Kp treatment. (C) Amplitude and period of calcium oscillation before and after Kp treatment (n = 57 from 2 batches, *** $p < 0.001$ by Student's t-test).

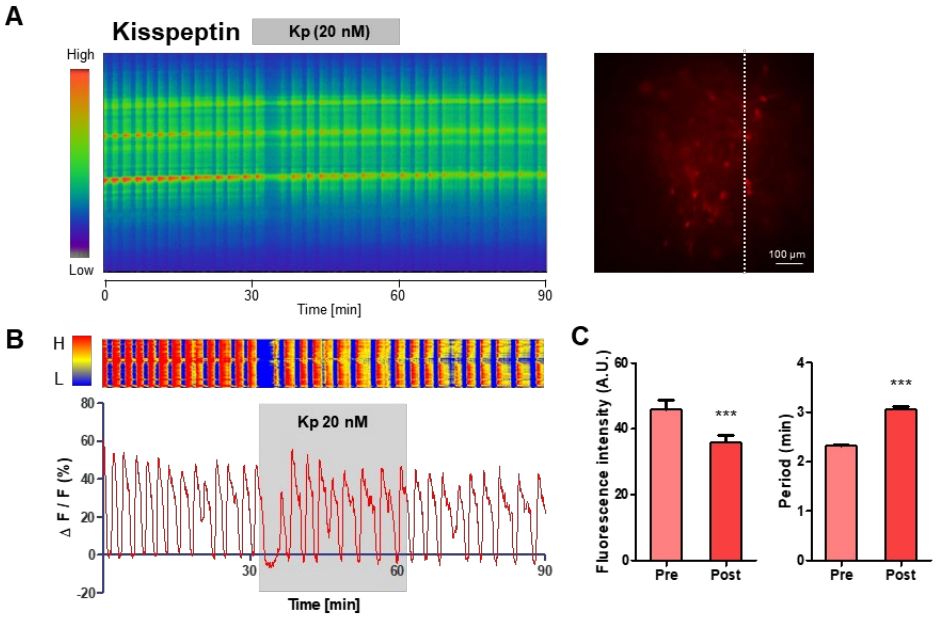

A
Kisspeptin
Kp (20 nM)
High
Low
0
30
60
90
Time [min]
100 µm
B
H
L
Kp 20 nM
Δ F / F (%)
80
60
40
20
0
-20
30
60
90
Time [min]
C
Fluorescence intensity (A.U.)
60
40
20
0
***
Pre
Post
Period (min)
4
3
2
1
0
***
Pre
Post

Figure 22. Effect of NK3R agonist treatment on ARN kisspeptin neuron-specific calcium oscillations. (A) Schematic model showing KNDy neurons, representative image, and change in fluorescence intensity of RGECO from the time-lapse representation of the indicated vertical line with senktide (Senk, 20 nM) treatment. Scale bar: 100 μm. (B) Raster plot and quantified graph of calcium oscillation with Senk treatment. (C) Amplitude and period of calcium oscillation before and after Senk treatment (n = 59 from 2 batches, *** $p < 0.001$ by Student's t-test).

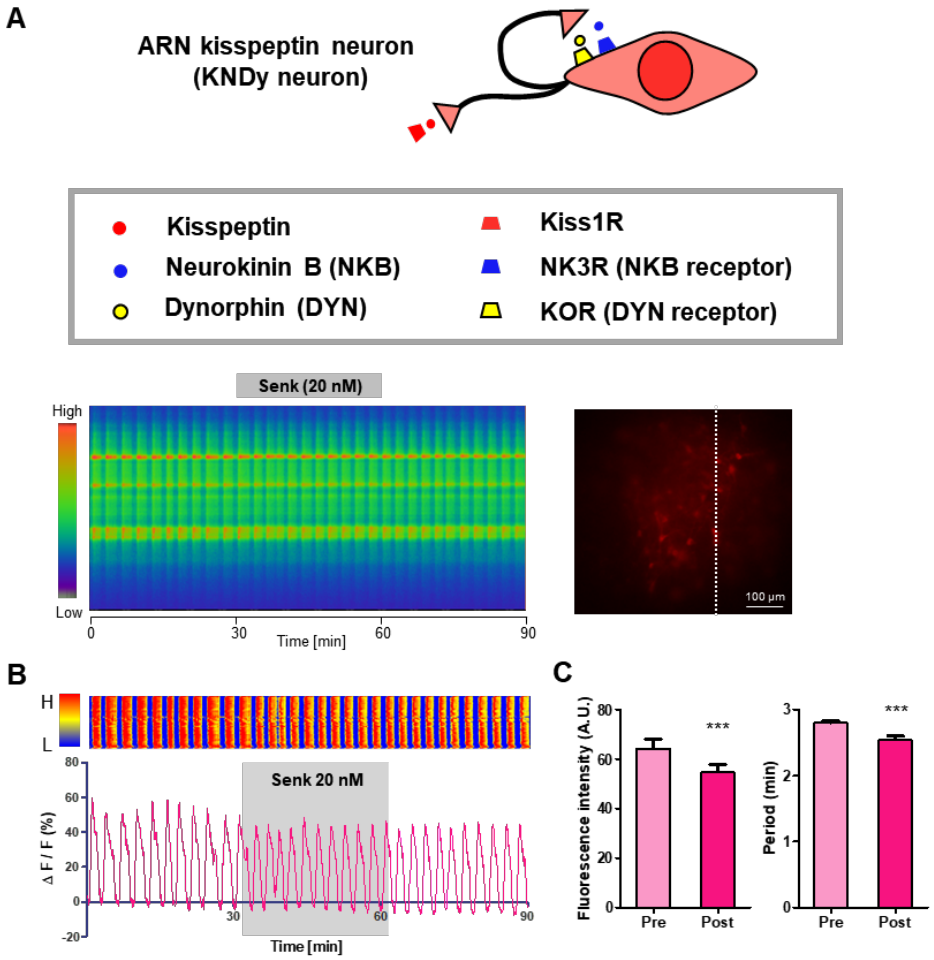

A
ARN kisspeptin neuron
(KNDy neuron)
Kisspeptin
Neurokinin B (NKB)
Dynorphin (DYN)
Kiss1R
NK3R (NKB receptor)
KOR (DYN receptor)
Senk (20 nM)
High
Low
0
30
60
90
Time [min]
100 µm
B
H
L
Senk 20 nM
Δ F / F (%)
80
60
40
20
0
-20
30
60
90
Time [min]
C
Fluorescence intensity (A.U.)
80
60
40
20
0
***
Pre
Post
Period (min)
3
2
1
0
***
Pre
Post

Figure 23. Effect of dynorphin treatment on ARN kisspeptin neuron-specific calcium oscillations. (A) Schematic model showing KNDy neurons, representative image, and change in fluorescence intensity of RGECO from the time-lapse representation of the indicated vertical line with dynorphin (Dyn, 100 nM) treatment. Scale bar: 100 μm. (B) Raster plot and quantified graph of calcium oscillation with Dyn treatment. (C) Amplitude and period of calcium oscillation before and after Dyn treatment (n = 89 from 2 batches, * $p < 0.05$ by Student's t-test)

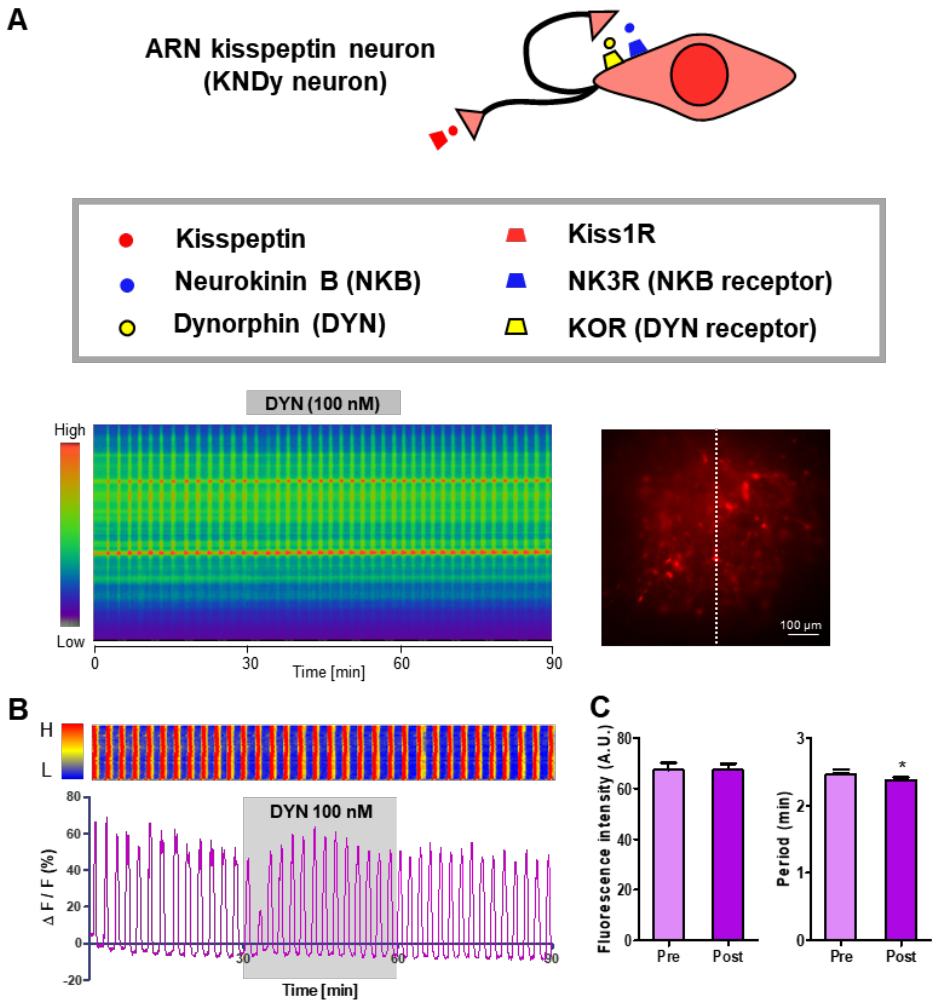

A
ARN kisspeptin neuron
(KNDy neuron)
Kisspeptin
Neurokinin B (NKB)
Dynorphin (DYN)
Kiss1R
NK3R (NKB receptor)
KOR (DYN receptor)
DYN (100 nM)
High
Low
0
30
60
90
Time [min]
100 µm
B
H
L
DYN 100 nM
Δ F / F (%)
Time [min]
C
Fluorescence intensity (A.U.)
Pre
Post
Period (min)
*

Figure 24. Role of NMDA receptor-mediated neurotransmission on ARN kisspeptin neuron-specific calcium oscillation. (A) Representative image and change in fluorescence intensity of RGECO from the time-lapse representation of the indicated vertical line with NMDA receptor antagonist (D-AP5, 50 μM) application. Scale bar: 200 μm. (B) Raster plot and quantified graph of calcium oscillation with D-AP5 treatment. (C) Amplitude and period of calcium oscillation before and after D-AP5 treatment (n = 25 from 2 batches, *** $p < 0.001$ by Student's t-test).

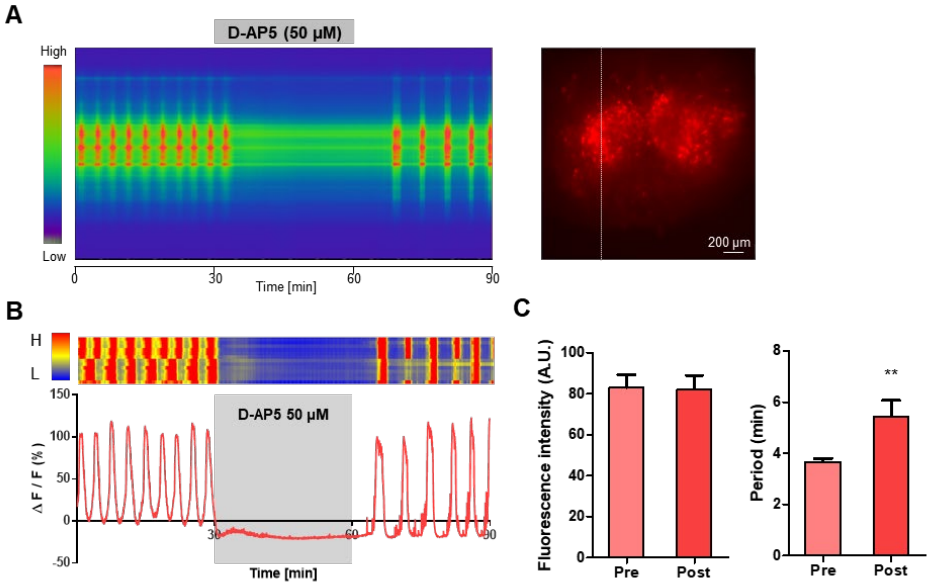

A
D-AP5 (50 µM)
High
Low
0
30
60
90
Time [min]
200 µm
B
H
L
150
100
50
0
-50
ΔF / F (%)
D-AP5 50 µM
30
60
90
Time [min]
C
Fluorescence intensity (A.U.)
100
80
60
40
20
0
Pre
Post
Period (min)
8
6
4
2
0
**
Pre
Post

Figure 25. Role of $GABA_A$ receptor-mediated neurotransmission on ARN kisspeptin neuron-specific calcium oscillation. (A) Representative image and change in fluorescence intensity of GCaMP from the time-lapse representation of the indicated vertical line with $GABA_A$ receptor antagonist (BIC, 10 μM) application. Scale bar: 200 μm. (B) Raster plot and quantified graph of calcium oscillation with BIC treatment. (C) Amplitude and period of calcium oscillation before and after BIC treatment (n = 128 from 3 batches, *** $p < 0.001$ by Student's t-test).

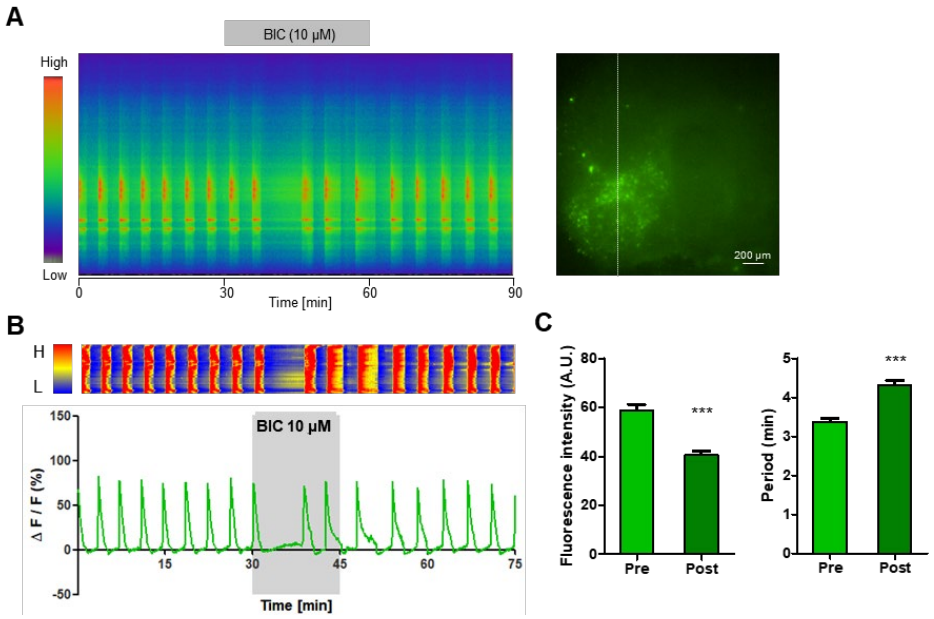

A
BIC (10 µM)
High
Low
0
30
60
90
Time [min]
200 µm
B
H
L
BIC 10 µM
Δ F / F (%)
150
100
50
0
-50
15
30
45
60
75
Time [min]
C
Fluorescence intensity (A.U.)
80
60
40
20
0
***
Pre
Post
Period (min)
5
4
3
2
1
0
***
Pre
Post

Figure 26. Co-culture of slice containing GnRH neurons with slice containing kisspeptin neurons. (A) Experimental scheme for co-culture of GnRHp-dsLuc mice. The organotypic slice was obtained from an 18° angled section to contain the POA and ARN region in the co-culture. (B) Real-time bioluminescence imaging scheme in a custom imaging system, Circadian 700B, without LED stimulation. (C) Scattered GnRH neurons with luminescence signals. Scale bar: 200 μm. (D) Kisspeptin neurons identified from angled slice culture. Scale bar: 20 μm.

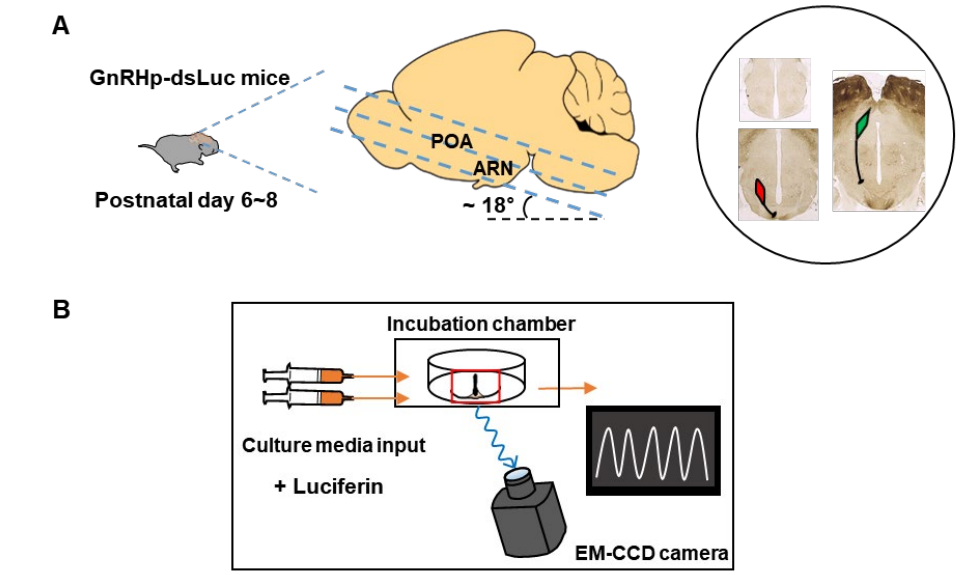

A
GnRHp-dsLuc mice
Postnatal day 6~8
POA
ARN
~ 18°
B
Incubation chamber
Culture media input
+ Luciferin
EM-CCD camera
C
200 μm
Luminesence
D
NeuroTrace
Kisspeptin
Merged
20 μm

Figure 27. Sporadic oscillations of GnRH transcription activity under basal conditions. Individual profile of GnRH promoter activity monitored with luciferase activity.

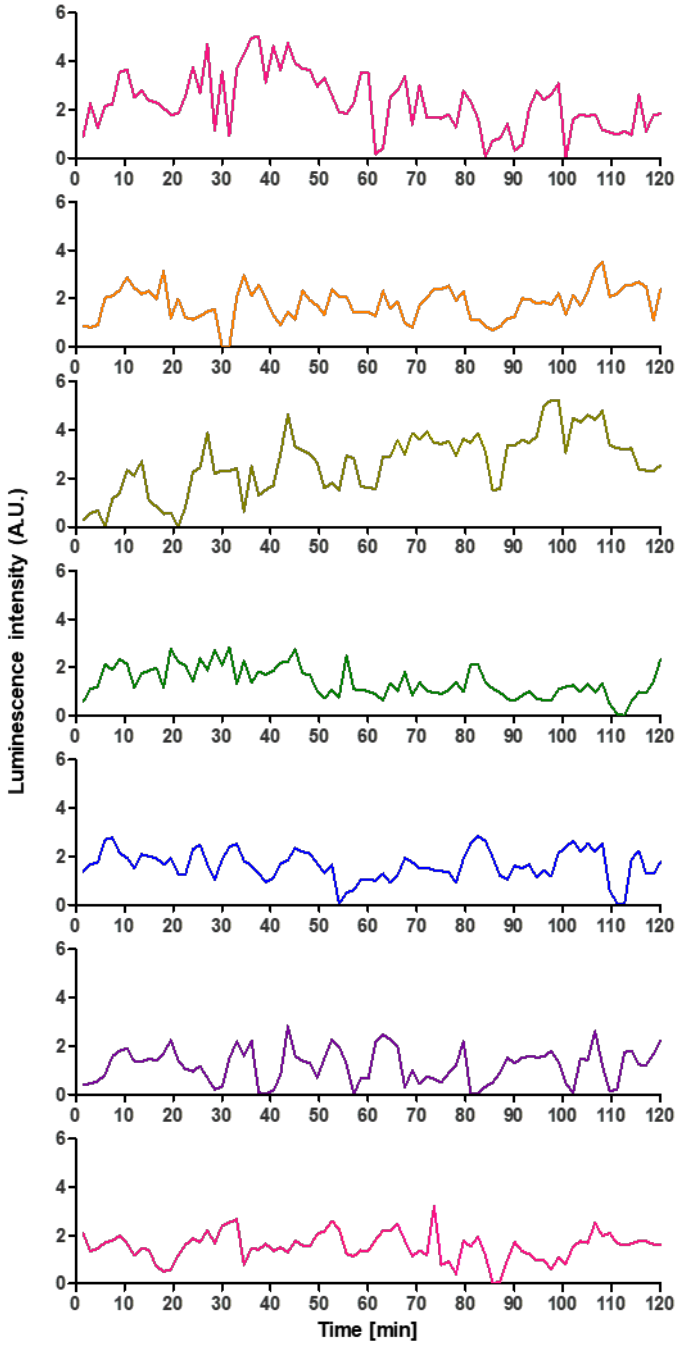

Luminescence intensity (A.U.)
Time [min]
0
2
4
6
0 10 20 30 40 50 60 70 80 90 100 110 120

Figure 28. GnRH neurons in slice respond to pulsatile kisspeptin treatment. Raster plot (upper panel) and quantified graph (lower panel) of GnRHp-dsLuc with intermittent kisspeptin (Kp, 10 nM) treatment to the angled slice.

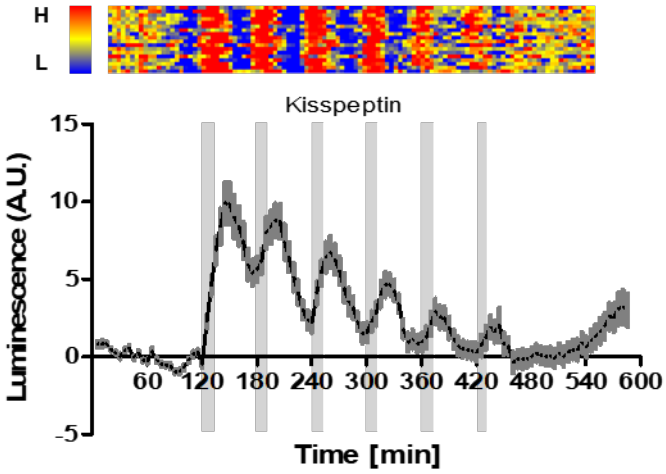
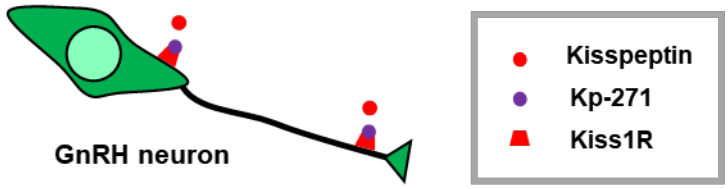
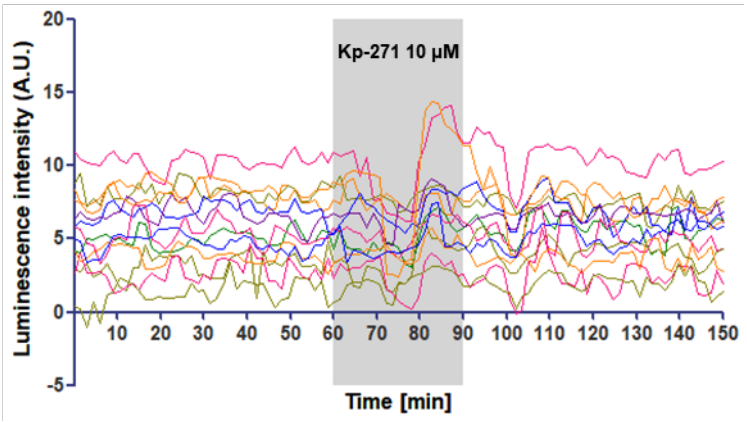

H
L
Kisspeptin
15
10
5
0
-5
Luminescence (A.U.)
60
120
180
240
300
360
420
480
540
600
Time [min]

Figure 29. Effect of kisspeptin receptor antagonist on baseline GnRH transcript activity. (A) Model showing pharmacological blockade of kisspeptin receptor signaling. (B) Change in GnRHp-dsLuc activity with kisspeptin receptor antagonist Kp-271 (10 μM) treatment. Each line represents an individual cell.

A


GnRH neuron
Kisspeptin
Kp-271
Kiss1R

B


Kp-271 10 µM
Luminescence intensity (A.U.)
Time [min]
20
15
10
5
0
-5
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150

DISCUSSION

The present study demonstrated that ARN kisspeptin neurons exhibit self-sustained and synchronized calcium oscillation in organotypic brain slice cultures. Synchronized pulsatile calcium oscillations were specific to kisspeptin neurons in the ARN, compared to the pan-neuronal population in the region. I observed that the voltage-gated sodium and potassium channels contribute to regulating the synchronized oscillation in ARN kisspeptin neurons, indicating that communication at a network level is required to maintain the synchronous calcium oscillation. Chemogenetic modulation of ARN kisspeptin neurons revealed that a network within the ARN kisspeptin neuronal population is involved in mediating the synchronized oscillation. Although not as robust as the effects of pharmacological modulation of ion channels and the chemogenetic modulation, kisspeptin lengthened the oscillation period while senktide decreased and DYN also slightly decreased the oscillation period. I also observed that both NMDA and $GABA_A$ receptor-dependent signaling contributed to maintaining the synchronized calcium oscillation in ARN kisspeptin neurons, and the effect of antagonizing NMDA receptor was more crucial. The present study demonstrated, for the first time, that synchronized calcium oscillation is present in ARN kisspeptin neurons *ex vivo* postnatally.

ARN kisspeptin neurons can generate ultradian calcium oscillation *ex vivo*

Recently, ARN kisspeptin neurons were reported to exhibit synchronized calcium oscillations with a period of approximately 9 min using fiber photometry (Clarkson et al., 2017) that is longer than our observation (3 min). One of the differences between the two studies is the model, with adult mice used *in vivo* (Clarkson et al., 2017) compared to postnatal mice used *ex vivo*. There are two plausible explanations for the difference between the two results. First, there are differences in the organization of gonadal steroid produced by the preparation of the animals. A previous study demonstrated that, when gonadal steroid input is abolished, the calcium episode becomes more frequent along with time after gonadectomy in male mice (Han et al., 2019). The organotypic slices in the present study are prepared from neonatal mice and then maintained for more than 20 days *in vitro* in isolated cultures without inputs during the postnatal period. It is plausible that the ARN kisspeptin neuronal population may become mature along with sexual development establishing steroid milieu, even though kisspeptin-expressing neurons are found in the ARN from the embryonic stage and already communicate with GnRH neurons (Kumar et al., 2015). Second, kisspeptin neurons in the AVPV start to express from postnatal day 25 (Clarkson and Herbison, 2006), and the innervation between AVPV and ARN kisspeptin neurons was observed in 2-4 month male and female mice (Yip et al., 2015). It is probable that maturation and communication between the two regional

populations in adult mice alters the regulation of ARN neurons compared to the neonatal state.

ARN kisspeptin neurons are regulated by several neural signaling factors

Continuous local neuronal transmission is essential for the synchronized calcium oscillation in ARN kisspeptin neurons the as revealed by the ion channel blocker experiments. Also, the results from chemogenetic experiments suggest that the inputs within ARN kisspeptin neurons could be involved in generating the synchronized calcium oscillation. Previous studies reported that NKB stimulated, while DYN inhibited ARN kisspeptin neuron activity (Moore et al., 2018). In the present study, senktide decreased the period of calcium oscillation after treatment, whereas DYN temporally inhibited the oscillation and slightly decreased the period after treatment, suggesting that they can also modulate the frequency of ARN kisspeptin neuron-specific calcium oscillation. On the other hand, it is possible that other neuronal populations expressing Kiss1R in the ARN (Higo et al., 2017) could play an additional role in modulating the synchronized calcium oscillation. This reference could support our findings of increased period and decreased amplitude of synchronized calcium oscillation. Hypothalamic ARN is involved in numerous physiological functions and is composed of diverse cell types as revealed by single-cell RNA-seq technology (Campbell et al., 2017). For instance, proopiomelanocortin (POMC) neurons in the ARN, which mediate

satiety, express Kiss1R and are known to receive input from ARN kisspeptin neurons (Higo et al., 2017; Nestor et al., 2016). Interestingly, kisspeptin is also suggested to activate some receptors of RFamide peptides, a family of neuropeptides characterized by arginine-phenylalanine-amide at the C-terminal (Liu and Herbison, 2016). For example, RFamide-related peptide 3 receptor is expressed in the ARN of mice (Poling et al., 2013), indicating that kisspeptin may also act through RFamide peptide receptors to modulate the oscillation.

Inhibition of NMDA receptor with D-AP5 immediately, but transiently suppressed the calcium oscillation. Notably, about 90% of ARN kisspeptin neurons express mRNAs that encode for vesicular glutamate transporter 2, which reflects excitatory neurons that release glutamate (Nestor et al., 2016). In a previous study, when ARN kisspeptin neurons were stimulated, the glutamatergic fast excitatory postsynaptic potential was detected in the contralateral side (Qiu et al., 2016). Based on our results and these references, it may be concluded that glutamatergic transmission mediated by NMDA receptor among ARN kisspeptin neurons could be involved in modulating the synchronized calcium oscillation. On the other hand, inhibiting $GABA_A$ receptor transmission increased the oscillation period in our results. In a previous study, GABA application to adult ARN slice silenced the firing of kisspeptin neurons (Gottsch et al., 2011). Yet, GABA, known as an inhibitory transmitter, can exert excitatory actions in immature neurons with higher intracellular chloride ion concentration, that gradually switch to inhibitory

action in the course of development (Ben-Ari et al., 2007). This GABAergic regulation on the synchronized calcium oscillation of ARN kisspeptin neurons has a potential origin in the ARN region. Most of the agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons in the ARN are reported as GABAergic neurons (Marshall et al., 2017). ARN kisspeptin neurons arise from *Pomc*-expressing progenitors, along with POMC and AgRP/NPY neurons that have an antagonistic function in regulating energy homeostasis, suggesting a possible link between nutrition-sensing and reproductive development (Sanz et al., 2015).

The impact of the kisspeptin neurons on the GnRH pulse generator

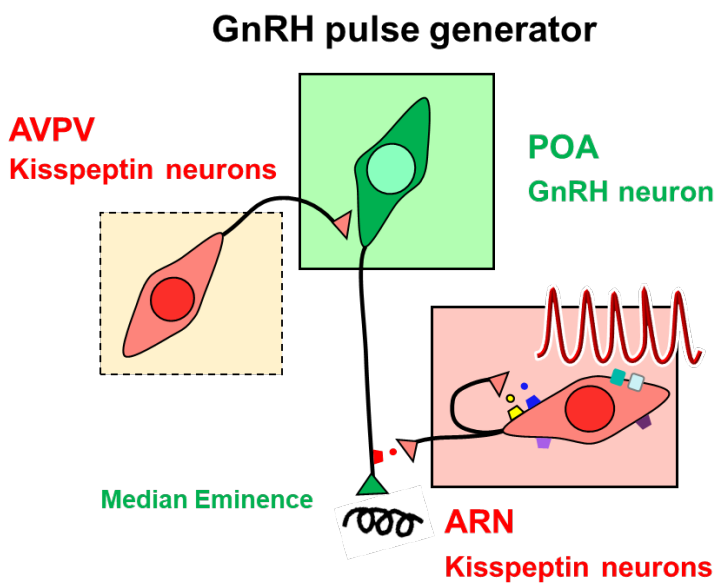
In an attempt to examine the relation between kisspeptin and GnRH neurons, I monitored GnRH gene expression as an index of the GnRH pulse generator, since kisspeptin administration induced pulsatile transcript activity as well as secretion (Choe et al., 2013). In the present study, GnRH promoter activity observed by luciferase activity did not seem to be in correlation with the calcium oscillation with an approximately 3 min period observed in ARN kisspeptin neurons. Nevertheless, Kp-271, a kisspeptin receptor antagonist, treatment partially decreased the basal GnRHp-dsLuc activity, suggesting the involvement of kisspeptin signaling in generating the endogenous GnRH gene expression. However, the exact relation between the spontaneous calcium oscillation of the ARN kisspeptin neurons and the pulsatile rhythm in GnRH neurons remains to be explored. There are differences in temporal

dimensions exist in the calcium activity exhibited in GnRH neurons, pulsatile secretion of GnRH, and the stimulus required to activate GnRH neurons. In previous studies, GnRH neurons exhibited approximately 8 sec of calcium transients recorded in acute slices of PeriCam, a transgenic mouse line expressing genetically encodable calcium indicator (Constantin et al., 2012), whereas the GnRH pulsatility in secretion was at least about 20 min in GT1 cells as well as in the *ex vivo* median eminence superfusion (Kim and Ramirez, 1985; Martínez de la Escalera et al., 1992). Moreover, for optogenetic manipulation of GnRH neurons, 10 Hz stimulation for 2 min was sufficient for generating LH pulses (Campos and Herbison, 2014). Therefore, investigating the relation of ARN kisspeptin neuron-specific calcium oscillation to kisspeptin secretion and, moreover, to GnRH secretion would be required to elucidate the role of the ARN kisspeptin neuronal calcium oscillations in the GnRH pulse generator.

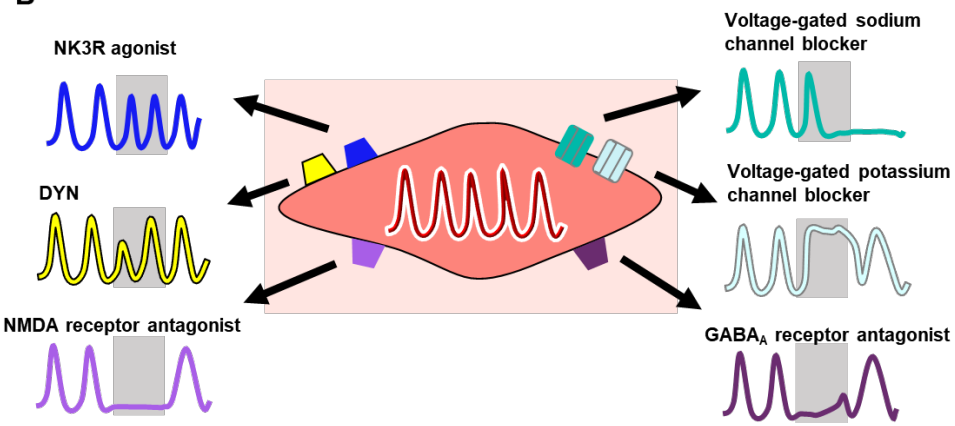
In summary, our study demonstrated that postnatal ARN kisspeptin neurons can generate a self-sustained and synchronized ultradian rhythm *ex vivo*. ARN kisspeptin neuron-specific calcium oscillation was regulated by intra-ARN inputs including ARN kisspeptin neurons. The present study suggests the ARN kisspeptin neurons as an ultradian rhythm generator contributing to the GnRH pulse generator (Fig. 30).

Figure 30. Diagram of ARN kisspeptin neuron-specific calcium oscillation and the impact on the GnRH pulse generator. (A) GnRH pulse generator model and the effects of kisspeptin. ARN kisspeptin neurons can generate self-sustained, synchronized ultradian rhythm *ex vivo*. Kisspeptin signaling have a role in maintaining basal GnRH transcription. (B) Synchronized calcium oscillation in ARN kisspeptin neurons is regulated by various signaling factors. Summarized effects of pharmacological treatments.

A


GnRH pulse generator
AVPV
Kisspeptin neurons
POA
GnRH neuron
Median Eminence
ARN
Kisspeptin neurons
Kisspeptin
Neurokinin B (NKB)
Dynorphin (DYN)
Voltage-gated sodium channel
Voltage-gated potassium channel
Kiss1R
NK3R (NKB receptor)
KOR (DYN receptor)
NMDA receptor
GABA$_A$ receptor

B


NK3R agonist
DYN
NMDA receptor antagonist
Voltage-gated sodium channel blocker
Voltage-gated potassium channel blocker
GABA$_A$ receptor antagonist

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국문 초록

맥동적으로 분비되는 시상하부의 성선자극호르몬 분비호르몬(gonadotropin-releasing hormone, GnRH)은 발생과 생식 발달을 관장하는 데 중요한 인자이다. 또한 시상하부 내 궁상핵(arcuate nucleus, ARN)의 kisspeptin 신경세포는 포유류 생식 조절의 최종 공통 경로인 GnRH 신경세포를 조절하는 중요한 구성 요소로 알려져 있다. 40여년 전 신경내분비학의 시초를 열었던 GnRH의 발견 이후로 많은 연구가 진행되었지만, 'GnRH 맥동 발진기'의 작동 기전은 여전히 밝혀지지 않았다. 특히 GnRH의 상위 조절자로 알려진 ARN kisspeptin 신경세포가 자체적으로 맥동을 발생시킬 수 있는지, 단순히 신경 정보를 전달하는 것인지, 등 자세한 기작은 아직 완전히 밝혀지지 않고 있다. 따라서 본 연구는 ARN kisspeptin 신경세포의 내재적인 리듬과, 그의 신경 조절을 연구하고자 하였다. 이를 위해 *Kiss1*-IRES-Cre 생쥐의 두뇌 절편 배양에 유전자 재조합 칼슘 센서를 발현시키고, 실시간 형광 및 인광 이미징 장비를 이용하여 ARN kisspeptin 신경세포로부터 칼슘 역학을 관찰하였다. 또한 신경 조절 연구를 위해 약리학적 접근법을 이용하여 이온 채널 차단제, ARN kisspeptin 신경세포로부터 방출되는 신경펩타이드와 작용제, 그리고 NMDA 및 GABA 수용체 억제제를 처리하며 그 효과를 조사하였다. 특히, kisspeptin 신경세포 특이적으로 활성을 조절하기 위해 화학유전학(DREADD) 시스템을 이용하여 ARN kisspeptin 신경세포군의 칼슘

진동 발생에 대한 기여도를 관찰하였다. 끝으로, GnRH 프로모터에 의해 인광 리포터를 발현하는 생쥐의 두뇌 절편 배양을 사용하여 GnRH 맥동 발진기와의 관계를 조사하였다. ARN kisspeptin 신경세포는 두뇌 절편 배양에서 약 3분 주기의 아일주기 리듬으로 강력하고 동기화된 칼슘 진동을 나타냈다. Kisspeptin 신경세포 특이적으로 관찰된 칼슘 진동은 활동 전위에 의해 매개되는 시냅스 전달에 의존적이었고, ARN kisspeitn 신경세포에서 자가 분비되는 신경펩타이드에 의해 어느 정도 영향을 받았으며, NMDA 및 GABA 수용체에 의해 매개되는 신경 전달의 조절을 받았다. Kisspeptin 신경세포를 포함하는 두뇌 절편과 GnRH 신경세포를 포함하는 두뇌 절편의 합동 배양에서 외부 kisspeptin 처리에 의해 GnRH 유전자 발현이 동기화 되었으며, kisspeptin 수용체 억제제 처리에 의해 GnRH 유전자 발현이 부분적으로 감소하였다. 결론적으로, 본 연구는 ARN kisspeptin 신경세포가 생체 외에서 동기화되고 자체적으로 유지되는 칼슘 진동을 생성할 수 있음을 직접적으로 규명하였다. 특히 이러한 칼슘 진동이 kisspeptin 신경세포 자체를 포함하여, ARN 영역 내의 여러 요인에 의해 조절 받는다는 사실을 확인하였다. 아울러, kisspeptin을 통한 신경 전달이 GnRH 신경세포 전사 조절에 영향을 미치며, 맥동 발진기에 기여하고 있음을 제시한다.

감사의 글

연구실에 처음 들어선 날이 아직도 생생하게 기억이 나는데, 이렇게 박사학위논문에 감사의 글을 작성하니 감회가 새롭습니다. 돌아보면 아쉬운 부분도 물론 있지만, 짧지 않은 시간 동안 많은 것을 배울 수 있었습니다. 지금에 이르기까지 많은 분들의 도움을 받았습니다. 먼저, 항상 모범을 보이시면서 조언과 격려로 이끌어주신, 저의 멘토이신 김경진 교수님께 진심으로 감사드립니다. 앞으로 선생님 가르침을 새기며 부족한 부분 더 발전해 나가겠습니다. 또한 바쁘신 중에도 논문 심사를 맡아 주신 정용근 교수님, 박동은 교수님, 최석우 교수님, 그리고 최한경 교수님께도 깊이 감사드립니다. 많은 것을 가르쳐주신 연구실의 선배님들께도 감사 인사를 드리고 싶습니다. 연구를 진행하는 중에 상의 드릴 때마다 흔쾌히 도움을 주신 이석원 박사님, 손기훈 교수님과 정수영 교수님, 실험의 기초부터 차근차근 알려주신 사수 김희대 박사님, 늘 차분하게 조언해 주신 이은정 박사님, 대구까지 함께 오면서 많은 의지가 되어 주신 전승국 박사님과 다른 선배님들께도 감사드립니다. 그리고 연구실 동기로서 오랫동안 함께 있어준 김정아 박사와 학위 심사 발표 자리까지 와주신 김은숙 아주머니께도 이 자리를 빌어 감사드립니다. 또 DGIST 에서 부족한 동생을 선배라고 배려하며 도와준 인아언니, 일이 많을 때 선뜻 손을 내밀어준 상원오빠, 실험실 막내로 묵묵히 잔일을 거들어준 교진이, 바쁜 중에도 언제나 도와준 최미정 연구원과, 정신 없는 저를 잘 챙겨준 최한경 교수님 연구실원들까지, 모두가 있어 다방면으로 힘을 받았습니다. 학위과정 동안 기쁘고 보람되는 일들과 힘들고 인내를 요하는 일들이 공존했는데, 여러 분들이 함께 있어 지금의 제가 있다고 생각합니다. 마지막으로, 지금까지 한결같은 관심과 격려로 응원해준 가족들과 김재연 박사에게도 감사와 사랑을 전합니다.