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Opioidergic pathways and kisspeptin in the regulation of female reproduction in mammals

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Endogenous opioid peptides have attracted attention as critical neuropeptides in the central mechanism regulating female reproduction ever since the discovery that arcuate dynorphin neurons that coexpress kisspeptin and neurokinin B (NKB), which are also known as kisspeptin/neurokinin B/dynorphin (KNDy) neurons, play a role as a master regulator of pulsatile gonadotropin-releasing hormone (GnRH) release in mammals. In this study, we first focus on the role of dynorphin released by KNDy neurons in the GnRH pulse generation. Second, we provide a historical overview of studies on endogenous opioid peptides. Third, we discuss how endogenous opioid peptides modulate tonic GnRH/gonadotropin release in female mammals as a mediator of inhibitory internal and external cues, such as ovarian steroids, nutritional status, or stress, on reproduction. Then, we discuss the role of endogenous opioid peptides in GnRH surge generation in female mammals.

KEYWORDS

endogenous opioid peptides, dynorphin, β -endorphin, enkephalin, GnRH pulse generator, GnRH surge generator, KNDy neurons, *Kiss1*

Introduction

One of the most important findings on the role of endogenous opioid peptides in female reproduction over the last two decades is that dynorphin neurons in the hypothalamic arcuate nucleus (ARC) are involved in gonadotropin-releasing hormone (GnRH) pulse generation. More specifically, a majority of ARC dynorphin neurons coexpress kisspeptin and neurokinin B (NKB); thus, the neurons are also referred to as kisspeptin/neurokinin B/dynorphin (KNDy) neurons and act as master regulators of pulsatile GnRH release in mammals (Lehman et al., 2010a; Maeda et al., 2010; Okamura et al., 2013; Uenoyama et al., 2014, 2021b; Goodman et al., 2018; Moore et al., 2018; Ikegami et al., 2021; Nagae et al., 2021; Tsukamura, 2022). GnRH is intermittently secreted in the pituitary portal vessel (Clarke and Cummins, 1982; Moenter et al., 1992) and controls the tonic (pulsatile)

release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior lobe of the pituitary gland. The tonic release of LH and FSH governs follicular development and corpus luteum function in the ovaries of female mammals. GnRH pulses are fundamental for reproduction in female mammals as a pioneer study demonstrated that circulating LH and FSH levels were maintained only when GnRH was applied in a pulsatile manner at physiological intervals in female rhesus monkeys after the blockade of endogenous GnRH release by a hypothalamic lesion (Belchetz et al., 1978). The neuronal circuit driving GnRH pulse generation has generally been termed the GnRH pulse generator (Lincoln et al., 1985; Maeda et al., 1995), and the intrinsic source of the generator has been a major enigma until very recently.

The present review mainly focuses on how endogenous opioid peptides regulate and/or modulate tonic GnRH/gonadotropin release, which is regulated by KNDy neurons, in female mammals. We also provide a historical overview of studies on endogenous opioid peptides and a summary of our recent understanding of the role of hypothalamic opioidergic neurons in the mechanism regulating female reproduction under normal and stressful conditions.

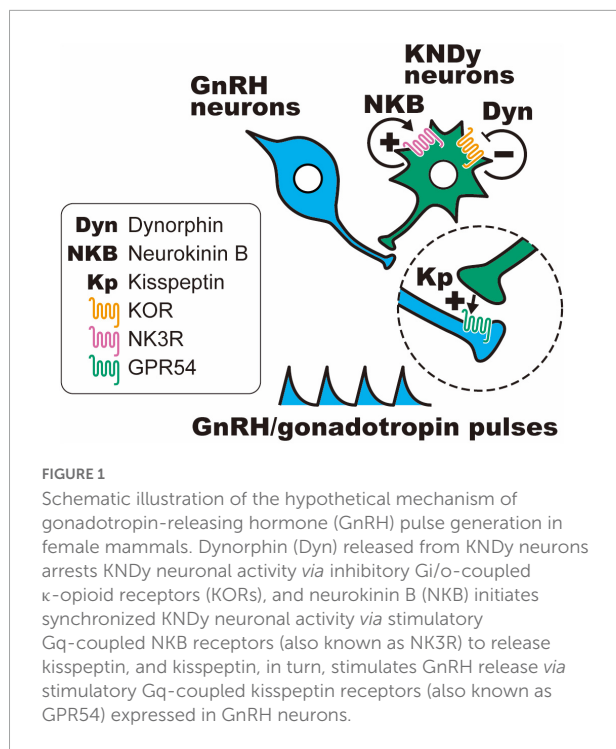
Kisspeptin/neurokinin B/dynorphin neurons as an intrinsic regulator of gonadotropin-releasing hormone pulses

Since the discovery of KNDy neurons, endogenous opioid peptides have attracted attention as critical neuropeptides in the central mechanism regulating female reproduction. Indeed, the discovery of KNDy neurons is one of the most exciting topics in reproductive neuroendocrinology over the last two decades. Using immunohistochemistry, Goodman and colleagues first demonstrated that dynorphin and NKB are largely coexpressed in a single population of ARC neurons in ewes (Foradori et al., 2006) and then revealed that kisspeptin is also expressed in the majority of the same neuronal population (Goodman et al., 2007). Importantly, Goodman and colleagues reported that none of the dynorphin neuronal populations located in the other hypothalamic regions, such as the paraventricular nucleus (PVN), supraoptic nucleus (SON), and preoptic area (POA), colocalized with NKB (Foradori et al., 2006) and kisspeptin (Goodman et al., 2007). Immediately thereafter, the coexpression of dynorphin, NKB, and kisspeptin in a population of ARC neurons was validated in several mammals, including goats (Wakabayashi et al., 2010), heifers (Hassaneen et al., 2016), rats (True et al., 2011; Murakawa et al., 2016), mice (Navarro et al., 2009; Ikegami et al., 2017), pigs (Harlow et al., 2021), and rhesus monkeys (Ramaswamy et al., 2010; True et al., 2017),

as summarized in our recent article (Uenoyama et al., 2021b). These findings implied the physiological importance of KNDy neurons for mammalian reproduction beyond the species, although colocalization of dynorphin in ARC kisspeptin/NKB neurons was not evident yet in humans (Hrabovszky et al., 2012, 2019).

Importantly, dynorphin receptors (i.e., κ -opioid receptors; KORs) were found in a majority of rat and ovine KNDy neurons (Weems et al., 2016; Tsuchida et al., 2020) and a portion of KNDy neurons in female mice (Navarro et al., 2009; Ikegami et al., 2017). In addition, the NKB receptors (also known as NK3R) were found in a majority of rodent and ovine KNDy neurons (Navarro et al., 2009; Amstalden et al., 2010; Ikegami et al., 2017). On the other hand, kisspeptin receptors (also known as GPR54) were found in the majority of GnRH neurons and were scarcely found in KNDy neurons of mice and rats (Herbison et al., 2010; Higo et al., 2016). These findings suggest that KNDy neurons communicate with each other by dynorphin-KOR and NKB-NK3R signaling in an autocrine/paracrine manner. As shown in Figure 1, the most plausible interpretation of the cellular mechanism regulating synchronized KNDy neuronal activity to drive GnRH pulses is as follows: we envisage that dynorphin released from KNDy neurons arrests KNDy neuronal activity *via* the inhibitory Gi/o-coupled KOR, NKB initiates synchronized KNDy neuronal activity *via* stimulatory Gq-coupled NK3R to release kisspeptin, and kisspeptin, in turn, stimulates GnRH release *via* stimulatory Gq-coupled GPR54 expressed in GnRH neurons (Navarro et al., 2009; Lehman et al., 2010a,b; Okamura et al., 2013; Uenoyama et al., 2014, 2021b; Goodman et al., 2018; Moore et al., 2018; Ikegami et al., 2021). Indeed, in female goats, the frequency of multiple unit activity (MUA) volleys, which were recorded in the vicinity of ARC KNDy neurons and accompanied by LH pulses, was decreased by the central administration of dynorphin and increased by the administration of a KOR antagonist (nor-binaltorphimine; nor-BNI) or NKB (Ohkura et al., 2009; Wakabayashi et al., 2010). These findings suggest that dynorphin-KOR signaling and NKB-NK3R signaling play a role in determining the frequency of GnRH pulse generator activities. Furthermore, central or peripheral administration of dynorphin or NK3R antagonists (SB223412 and SB222200) suppressed LH pulses, whereas KOR antagonists (nor-BNI and PF-4455242); NKB, an NK3R agonist (senktide); and kisspeptin stimulated LH pulses in several mammalian species, such as rodents (Gottsch et al., 2004; Irwig et al., 2004; Kinoshita et al., 2005; Messenger et al., 2005; Pheng et al., 2009; Navarro et al., 2011; Mostari et al., 2013; Ruiz-Pino et al., 2015) and ruminants (Messenger et al., 2005; Ohkura et al., 2009; Sakamoto et al., 2012; Tanaka et al., 2012; Goodman et al., 2013; Naniwa et al., 2013; Yamamura et al., 2015; Nakamura et al., 2017; Sasaki et al., 2019, 2020).

Recently, we rescued *Kiss1* (which is the gene that encodes kisspeptin) expression in ARC dynorphin/NKB neurons in



global *Kiss1*-knockout rats utilizing adeno-associated virus (AAV) vectors carrying *Kiss1* cDNA (Nagae et al., 2021). Rescuing *Kiss1* expression in 20–50% of ARC NKB neurons could recover pulsatile LH release and folliculogenesis up to the preovulatory follicles in global *Kiss1*-knockout female rats. These findings provide direct evidence that ARC KNDy neurons serve as an intrinsic source of the GnRH pulse generator in female mammals.

Brief history of studies on endogenous opioid peptides

Endogenous opioid peptides were found to be endogenous substances that produce the same analgesic effect as morphine, an opiate alkaloid derived from opium poppies (Brownstein, 1993; Snyder and Pasternak, 2003; Waldhoer et al., 2004; Gruber et al., 2007; Przewlocki, 2013; Devereaux et al., 2018). Opiate alkaloids have a long history of medicinal use since the time of ancient Greeks and Romans (Brownstein, 1993; Waldhoer et al., 2004; Gruber et al., 2007; Devereaux et al., 2018), and the active ingredient morphine was isolated in the middle of the 1800s (Devereaux et al., 2018). Morphine was introduced for pain treatment in the 1820s (Przewlocki, 2013; Devereaux et al., 2018), and then morphine, like original opiate alkaloids, was found to be an addictive drug (Brownstein, 1993; Przewlocki, 2013). In search of a safe analgesic, many opiate agonists and antagonists were developed (Brownstein, 1993; Gruber et al., 2007), and by the middle of the 1960s,

it was becoming clear that the analgesic effect of morphine and opiate agonists could be explained by the presence of specific receptors for the opiates in the brain (Snyder and Pasternak, 2003; Devereaux et al., 2018). In 1973, a radioreceptor assay with tritium-labeled and non-labeled opiate agonists or antagonists (Table 1) revealed the stereospecific binding of opiates, namely, opiate or morphine receptors, in rat brain homogenates (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). These findings implied the presence of endogenous opioid ligand(s) as neurotransmitters in the central nervous systems of mammals. In 1975, two pentapeptides, Tyr-Gly-Gly-Phe-Met (termed Met-enkephalin) and Tyr-Gly-Gly-Phe-Leu (termed Leu-enkephalin), were found in the pig brain as endogenous ligands for opiate or morphine receptors (Hughes et al., 1975a,b). It soon became obvious that the Met-enkephalin sequence was present on the N terminus of another endogenous opioid peptide, that is, β -endorphin, in 1976 (Birdsall and Hulme, 1976; Li and Chung, 1976). Subsequently, the Leu-enkephalin sequence was found at the N terminus of another endogenous opioid peptide, dynorphin, in 1979 (Goldstein et al., 1979, 1981). To date, these endogenous opioid peptides have been classified into three families and were reported to be derived from three distinct precursors encoded by *Pomc*, *Penk*, and *Pdyn* genes (Nakanishi et al., 1979; Kakidani et al., 1982; Noda et al., 1982; Akil et al., 1984; Froehlich, 1997; Benarroch, 2012). Figure 2 shows three precursors—preproopiomelanocortin, preproenkephalin, and preprodynorphin—of endogenous opioid peptides, such as β -endorphin, Met- and Leu-enkephalins, and the dynorphin family [dynorphin A, and α - and β -neoenkephalins, leuromorphin, and rimorphin (also known as dynorphin B)], respectively, in humans and rats.

TABLE 1 Representative opiate agonists and antagonists used in the radioreceptor assay.

References	Agonists	Antagonists
Pert and Snyder, 1973	Codeine	Naloxone
	Levorphanol	Levallorphan
	Methadone	Nalorphine
	Morphine	
	Pentazocine*	
	Propoxyphene	
Simon et al., 1973	Etorphine	Nalorphine
	Levorphanol	Naloxone
	Methadone	
	Morphine	
Terenius, 1973	Codeine	Naloxone
	Dihydromorphine	
	Heroin	
	Levorphanol	

*Partial agonist.

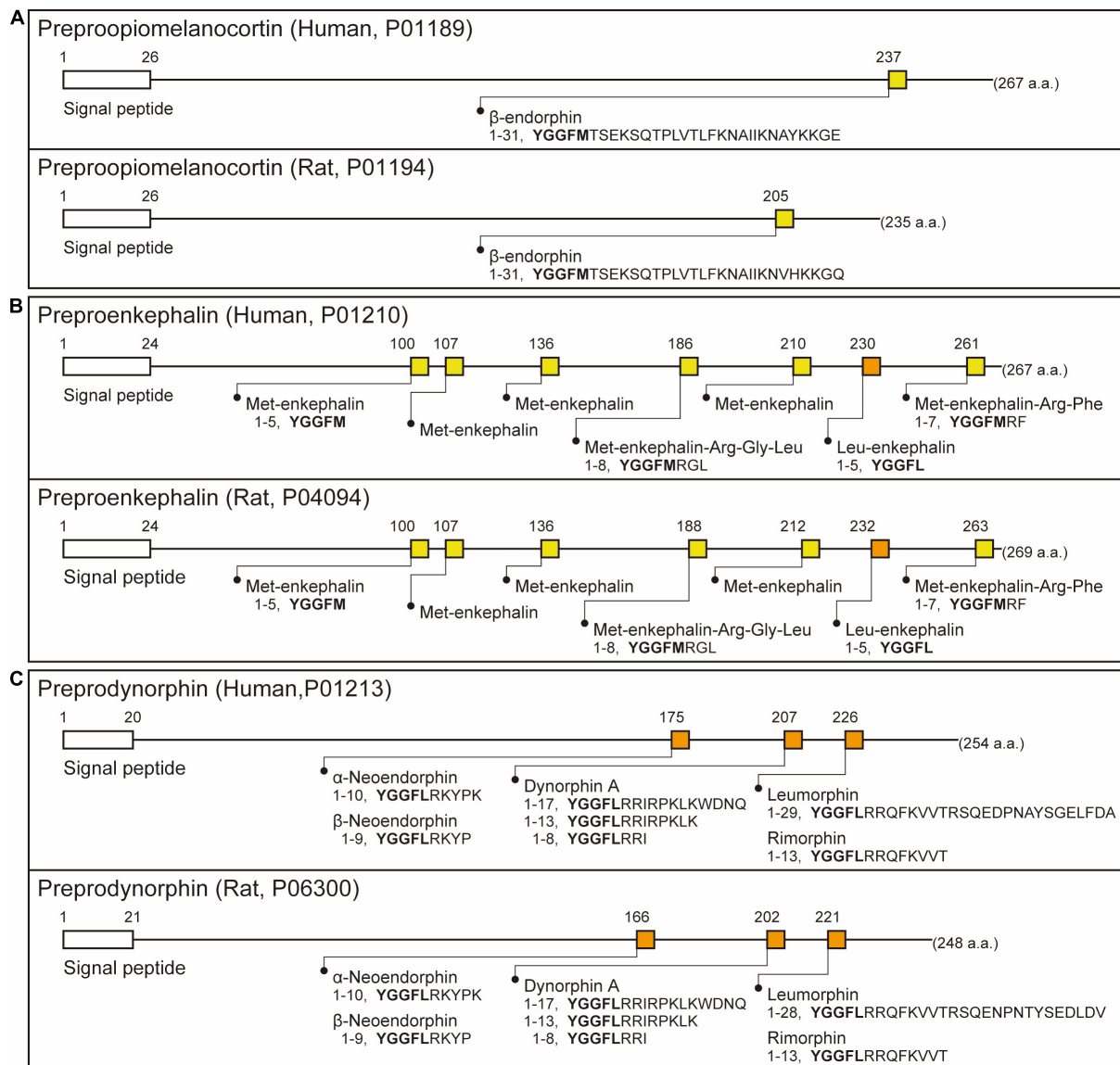


FIGURE 2

Schematic illustration of β -endorphin, Met- and Leu-enkephalins, and the dynorphin family (dynorphin A, α - and β -neoendorphins, leuomorphin, and rimorphin) in their precursors in humans and rats based on UniProtKB (<https://www.uniprot.org/uniprot/>). The precursors comprise a signal peptide at the N-terminal. (A) β -Endorphin consists of 31 amino acids cleaved from the precursor preproopiomelanocortin in humans and rats. Note that the five N-terminal amino acids (YGGMF, yellow squares) of β -endorphin, identical to Met-enkephalin, are commonly found in the mammals examined. (B) Met- (YGGMF, yellow squares) and Leu-enkephalins (YGGML, orange squares) consist of five amino acids cleaved from the precursor preproenkephalin. Note that human and rat preproenkephalin possess six Met-enkephalin and one Leu-enkephalin motifs, and two of six Met-enkephalin motifs are processed to eight or seven amino acid peptides (Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe). (C) Dynorphin A, α - and β -neoendorphins, leuomorphin, and rimorphin (also known as dynorphin B) consist of 8–28 amino acids cleaved from the single precursor preprodynorphin. Note that the five N-terminal amino acids (YGGML, orange squares) of all dynorphin family peptides are identical to Leu-enkephalin. The amino acid sequence of dynorphin A is identical among the mammals examined.

It is well known that β -endorphin, Met- and Leu-enkephalins, and the dynorphin family share three types of opioid receptors— μ -, δ -, and κ -opioid receptors (MOR, DOR, and KOR)—encoded by *Oprm1*, *Oprd1*, and *Oprk1* genes, respectively (Snyder and Pasternak, 2003; Waldhoer et al., 2004; Stein, 2016). As shown in Table 2, β -endorphin has been

reported to predominantly bind to both MOR and DOR with a similar affinity and with a lower affinity for KOR; Met- and Leu-enkephalins predominantly bind to the DOR with much higher affinity than the MOR and KOR; all dynorphin family peptides predominantly bind to the KOR, rather than the MOR and DOR (Yasuda et al., 1993; Raynor et al., 1994; Mansour et al., 1995b).

We should note that morphine was reported to predominantly bind to the MOR, followed by the KOR, with a low affinity for the DOR (Mansour et al., 1995b). These opioid receptors were cloned in rats and mice during the early 1990s (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993a,b; Fukuda et al., 1993; Li et al., 1993; Minami et al., 1993; Nishi et al., 1993; Thompson et al., 1993) and were found to belong to the large superfamily of seven-transmembrane G protein-coupled receptors. After the binding of an agonist, conformational changes of all three opioid receptors predominantly allow intracellular coupling of a heterotrimeric Gi/o protein (Connor and Christie, 1999; Waldhoer et al., 2004; Stein, 2016). Therefore, opioid receptor activation leads to inhibited adenylyl cyclase activity and reduced cAMP levels in target neurons (Connor and Christie, 1999; Waldhoer et al., 2004; Stein, 2016). In addition, opioid receptor activation leads to the opening of G protein-coupled inwardly rectifying K⁺ channels, thereby preventing neuronal excitation and/or propagation of action potentials of target neurons (Connor and Christie, 1999; Waldhoer et al., 2004; Stein, 2016). From these findings, it is well accepted that endogenous opioid peptides serve as inhibitory signals in the central nervous system *via* inhibitory Gi/o-coupled opioid receptors in mammals.

TABLE 2 Binding affinity and specificity of morphine and endogenous opioid peptides to opioid receptors.

Agents	Specificity	Species	References
Morphine	μ - >>> κ - \geq δ - μ - >> κ - >>> δ -	Mouse/rat ¹ Rat ²	Raynor et al., 1994 Mansour et al., 1995b
β -endorphin	δ - > κ - μ - \geq δ - >>> κ - μ - \geq δ - >> κ -	Mouse ³ Mouse/rat ¹ Rat ²	Yasuda et al., 1993 Raynor et al., 1994 Mansour et al., 1995b
Met-enkephalin	δ - >>> κ - μ - > δ - >>> κ - δ - > μ - >>> κ -	Mouse ³ Mouse/rat ¹ Rat ²	Yasuda et al., 1993 Raynor et al., 1994 Mansour et al., 1995b
Leu-enkephalin	δ - >>> κ - μ - \geq δ - >>> κ - δ - >>> μ - >>> κ -	Mouse ³ Mouse/rat ¹ Rat ²	Yasuda et al., 1993 Raynor et al., 1994 Mansour et al., 1995b
Dynorphin A ₁₋₁₇	κ - >> δ - κ - > δ - > μ - κ - > δ - > μ -	Mouse ³ Mouse/rat ¹ Rat ²	Yasuda et al., 1993 Raynor et al., 1994 Mansour et al., 1995b
α -neoendorphin	κ - >>> δ - κ - >> μ - \geq δ -	Mouse ³ Rat ²	Yasuda et al., 1993 Mansour et al., 1995b
β -neoendorphin	κ - > δ - > μ -	Rat ²	Mansour et al., 1995b
Leumorphin	κ - >> δ - \geq μ -	Rat ²	Mansour et al., 1995b
Rimorphin	κ - >>> δ - κ - > μ - \geq δ -	Mouse ³ Rat ²	Yasuda et al., 1993 Mansour et al., 1995b

>>>, more than 10 times; >>, more than 5 times; >, more than 2 times; \geq , less than 2 times.

¹Cloned mouse DOR and KOR and rat MOR cDNA were examined.

²Cloned rat MOR, DOR, and KOR cDNA were examined.

³Cloned mouse MOR, DOR, and KOR cDNA were examined.

Inhibitory roles of endogenous opioid peptides on tonic gonadotropin-releasing hormone/gonadotropin-releasing systems

Immediately after the isolation and characterization of the endogenous opioid peptides, the inhibitory effect of endogenous opioid peptides on pulsatile GnRH/gonadotropin release was intensively studied using the opioid receptor antagonist naloxone as a probe. As mentioned later in detail, peripheral and central administration of naloxone or other opioid antagonists facilitated tonic (pulsatile) LH release in female mammals at several stages of the reproductive cycle (Table 3) and under stressful conditions such as malnutrition and infection (Table 4). Thus, we envision that opioidergic neurons serve as mediators of inhibitory internal and external cues, such as ovarian steroids, nutritional status, or stress, on tonic GnRH/gonadotropin release in female mammals.

Involvement of endogenous opioid peptides in mediating the negative feedback action of ovarian steroids on tonic gonadotropin-releasing hormone/gonadotropin release

It is well established that the frequency of GnRH/gonadotropin pulses is fine-tuned by the negative feedback action of ovarian steroids such as estradiol-17 β (E2) and progesterone (P4) to keep circulating LH and FSH at proper levels to promote follicular development in the follicular phase of the estrous/menstrual cycle and maintain corpus luteum function in the luteal phase and pregnancy period (Nishihara et al., 1999; Herbison, 2020; Uenoyama et al., 2021a). Endogenous opioid peptides are suggested to be mediators of the negative feedback action of gonadal steroids on tonic GnRH/gonadotropin release in female mammals (summarized in Table 3). An intravenous (IV) injection of naloxone increased plasma LH levels during the late follicular (E2 dominant) and mid-luteal (P4 dominant) phases of the menstrual cycle, but not during the early follicular phase, in humans (Quigley and Yen, 1980). In addition, an IV injection of naloxone increased serum LH levels during the luteal phase, but not the follicular phase, in rhesus monkeys (Van Vugt et al., 1983). Likewise, subcutaneous (SC) injection of naloxone increased plasma LH levels in ovary-intact rats (Petraglia et al., 1984). An IV injection of naloxone stimulated LH secretion during the luteal phase, but not during the non-luteal phase, in ewes (Malven et al., 1984; Brooks et al., 1986). Furthermore, it has been noted that IV or SC administration of naloxone

TABLE 3 Effects of opioid receptor antagonists on tonic luteinizing hormone (LH) secretion in female mammals.

Antagonists	Receptors	Treatment routes	Effects	Species	Ovarian states	References
Naloxone	μ - > κ - >> δ -	i.v.	Stimulatory	Human	Late follicular phase	Quigley and Yen, 1980
		i.v.	Stimulatory	Human	Mid-luteal phase	Quigley and Yen, 1980
		i.v.	No effect	Human	Early follicular phase	Quigley and Yen, 1980
		i.v.	Stimulatory	Rhesus monkey	Luteal phase	Van Vugt et al., 1983
		i.v.	No effect	Rhesus monkey	Follicular phase	Van Vugt et al., 1983
		s.c.	Stimulatory	Rat	Ovary-intact	Petraglia et al., 1984
		s.c.	No effect	Rat	OVX	Petraglia et al., 1984
		i.v.	Stimulatory	Sheep	Luteal phase	Malven et al., 1984
		i.v.	No effect	Sheep	Non-luteal phase	Malven et al., 1984
		i.v.	Stimulatory	Sheep	Early and mid-luteal phase	Brooks et al., 1986
		i.v.	No effect	Sheep	Late-luteal phase	Brooks et al., 1986
		i.v.	No effect	Human	Post-menopausal	Reid et al., 1983
		3V	Stimulatory	Rat	Pregnant	Gallo, 1990
		MBH, POA	Stimulatory	Sheep	Luteal phase	Goodman et al., 2004
WIN44,441-3	κ -	i.v.	Stimulatory	Sheep	Luteal phase	Whisnant and Goodman, 1988
		i.v.	No effect	Sheep	Follicular phase	Whisnant and Goodman, 1988
		i.v.	Stimulatory	Sheep	Luteal phase	Yang et al., 1988
nor-BNI	κ -	3V	Stimulatory	Rat	Pregnant	Gallo, 1990
		3V	Stimulatory	Rat	OVX + low E2 ¹	Mostari et al., 2013
		3V	No effect	Rat	OVX	Mostari et al., 2013
MBH, POA	Stimulatory	Sheep	Luteal phase	Goodman et al., 2004		
PF-4455242	κ -	i.v., s.c.	Stimulatory	Goat	OVX + low E2 ²	Sasaki et al., 2019
Naloxonazine	μ -	POA	Stimulatory	Sheep	Luteal phase	Goodman et al., 2004
		MBH	No effect	Sheep	Luteal phase	Goodman et al., 2004
ICI 174864	δ -	3V	No effect	Rat	Pregnant	Gallo, 1990
Naltrindole	δ -	MBH, POA	No effect	Sheep	Luteal phase	Goodman et al., 2004

nor-BNI, nor-binaltorphimine.

¹Ovariectomized (OVX) rats treated with a diestrous level of E2. ²OVX goats treated with a luteal phase level of E2.

TABLE 4 Effects of opioid receptor antagonists on tonic luteinizing hormone (LH) secretion in female mammals under stressful conditions.

Antagonist	Receptors	Effects	Treatments	Species	Ovarian states	References
Naloxone	μ - > κ - >> δ -	Restored	Electric shock stress	Rat	Proestrus	Hulse and Coleman, 1983
		Restored	120-h fasting	Rat	OVX	Dyer et al., 1985
		Restored	48-h fasting	Rat	OVX + low E2 ¹	Cagampang et al., 1991
		Restored	hypoglycemia by insulin	Sheep	OVX	Clarke et al., 1990
		Restored	lipopolysaccharide	Cattle	OVX	Kujjo et al., 1995
		Restored	lipopolysaccharide	Rhesus monkey	OVX	Xiao et al., 2000
		Restored	CRH	Rhesus monkey	OVX	Gindoff and Ferin, 1987
		Restored	AVP	Rhesus monkey	OVX	Xiao et al., 1996
		Restored	CGRP	Rat	OVX	Bowe et al., 2005
β -funaltrexamine	μ -	Restored	CRH	Rat	OVX	Rivest et al., 1993
Naloxonazine	μ -	Restored	CRH	Rat	OVX	Rivest et al., 1993
CTOP	μ -	Restored	Glucoprivation by 2DG	Rat	OVX + low E2 ¹	Tsuchida et al., 2021
nor-BNI	κ -	No effect	CRH	Rat	OVX	Rivest et al., 1993
		Restored	Glucoprivation by 2DG	Rat	OVX + low E2 ¹	Tsuchida et al., 2020

CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂; CRH, corticotropin-releasing hormone; AVP, Arg-vasopressin; CGPR, calcitonin gene-related peptide; 2DG, 2-deoxy-D-glucose.¹OVX rats treated with a diestrous level of E2.

was unable to increase plasma LH levels in post-menopausal women (Reid et al., 1983) and ovariectomized (OVX) rats (Petraglia et al., 1984). In addition, naloxone administration into the third cerebroventricle (3V) facilitated LH pulses in rats during pregnancy (Gallo, 1990). These findings suggest that endogenous opioid peptides mediate the negative feedback action of E2 and P4 on pulsatile GnRH/LH release in female mammals. Furthermore, the local implant of crystalline naloxone into the mediobasal hypothalamus (MBH) or POA facilitated pulsatile LH release during the luteal phase in ewes (Goodman et al., 2004), suggesting that the MBH and POA, in which KNDy and GnRH neurons were found, respectively, in ewes (Lehman et al., 1986, 2010b; Goodman et al., 2007), could be possible action sites of endogenous opioid peptides to exert the negative feedback action of ovarian steroids on tonic GnRH/gonadotropin release. The expression of opioid receptors in KNDy and GnRH neurons will be discussed later.

Both KOR and MOR signaling are considered to mediate the negative feedback action of ovarian steroids on GnRH/gonadotropin release in female mammals. An IV injection of WIN44,441-3 (a specific KOR antagonist) facilitated LH pulses during the luteal phase of the estrous cycle (Whisnant and Goodman, 1988; Yang et al., 1988) but failed to facilitate LH pulses during the follicular phase in ewes (Whisnant and Goodman, 1988). Likewise, a 3V injection of nor-BNI (another KOR antagonist), but not ICI 174864 (a specific DOR antagonist), facilitated LH pulses in pregnant rats (Gallo, 1990). Our previous study showed that a 3V injection of nor-BNI stimulated the baseline levels of LH pulses in OVX rats treated with a diestrous level of E2, but not in OVX rats (Mostari et al., 2013). In addition, IV and SC injections of PF-4455242 (another KOR antagonist) facilitated LH pulses in OVX goats treated with a luteal phase level of E2 (Sasaki et al., 2019). Furthermore, the local implant of crystalline nor-BNI into the MBH or POA and the local implant of crystalline naloxonazine (a specific MOR antagonist) in the POA facilitated pulsatile LH release during the luteal phase in ewes (Goodman et al., 2004). By contrast, the local implant of crystalline naltrindole (a specific DOR antagonist) failed to facilitate pulsatile LH release during the luteal phase in ewes (Goodman et al., 2004). These results are consistent with the finding that naloxone was

reported to predominantly bind to the MOR, followed by the KOR, with a low affinity for the DOR (Mansour et al., 1995b). Taken together, these findings suggest that endogenous opioid peptides may mediate the negative feedback action of ovarian steroids *via* KOR signaling in the MBH and KOR and MOR signaling in the POA in female mammals.

Involvement of endogenous opioid peptides in mediating stress-induced suppression of tonic gonadotropin-releasing hormone/gonadotropin release

The frequency of GnRH/LH pulses is often suppressed under stressful conditions, such as malnutrition and infection (Chatterton, 1990; Tilbrook et al., 2000, 2002). Endogenous opioid peptides have attracted attention as mediators of the stress-induced suppression of GnRH/gonadotropin release in female mammals (summarized in Table 4). Previous studies have demonstrated that peripheral administration of naloxone blocks stress-induced LH suppression in several female mammals (Hulse and Coleman, 1983; Dyer et al., 1985; Clarke et al., 1990; Cagampang et al., 1991; Kujjo et al., 1995; Xiao et al., 2000). Concretely, an IV injection of naloxone blocked electric shock stress-induced LH suppression in proestrous female rats (Hulse and Coleman, 1983). Subcutaneous injections of naloxone blocked 48-h fasting-induced LH suppression in ovary-intact (Dyer et al., 1985) and OVX rats treated with a diestrous level of E2 (Cagampang et al., 1991). Our recent studies showed that the 3V administration of D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, another MOR antagonist) or nor-BNI restored suppression of LH pulses induced by peripheral or central injection of 2-deoxy-D-glucose (2DG, an inhibitor of glucose utilization) in OVX rats treated with a diestrus level of E2 (Tsuchida et al., 2020, 2021). Furthermore, IV administration of naloxone restored LH pulses that were suppressed by insulin-induced hypoglycemia in OVX ewes (Clarke et al., 1990), and IV injections of naloxone restored LH pulses that were suppressed by the administration of an

TABLE 5 Effects of opioid receptor antagonists on tonic luteinizing hormone (LH) secretion in pre-pubertal female mammals.

Antagonist	Receptors	Treatment routes	Effects	Species	Ovarian states	References
Naloxone	μ - > κ - >> δ -	s.c.	Stimulatory	Rat	Ovary-intact	Ieiri et al., 1980
		i.v.	Stimulatory	Sheep	Ovary-intact	Ebling et al., 1989
		i.v.	Stimulatory	Sheep	OVX + E2	Ebling et al., 1989
		i.v.	Stimulatory	Sheep	OVX	Ebling et al., 1989
		i.v.	Stimulatory	Sheep	OVX + E2	Wood et al., 1992
nor-BNI	κ -	i.p.	Stimulatory	Rat	Ovary-intact	Nakahara et al., 2013
		i.v.	Stimulatory	Sheep	OVX + E2	Lopez et al., 2016

endotoxin lipopolysaccharide in OVX heifers (Kujjo et al., 1995) and OVX rhesus monkeys (Xiao et al., 2000). Taken together, these findings suggest that endogenous opioid peptides mediate acute stress-induced suppression of GnRH/LH pulses under stressful conditions, such as malnutrition and infection, in female mammals.

It is well known that the stress response is mainly driven by the hypothalamic–pituitary–adrenal axis in mammals (Brooks and Challis, 1989; Senn et al., 1995; Bale and Vale, 2004; Papadimitriou and Priftis, 2009). Accumulating evidence has demonstrated that both corticotropin-releasing hormone (CRH) and Arg-vasopressin (AVP) neurons located in the PVN govern pituitary corticotrophin release and adrenal functions in response to various stressors (Brooks and Challis, 1989; Senn et al., 1995; Bale and Vale, 2004; Papadimitriou and Priftis, 2009). Thus, administration of CRH and AVP has been used to mimic stressful conditions to determine the role of opioids as mediators. As shown in Table 4, IV and lateral ventricle (LV) administration of naloxone restored CRH- and AVP-induced suppression of the frequency of LH pulses in OVX rhesus monkeys, respectively (Gindoff and Ferin, 1987; Xiao et al., 1996). These findings suggest that opioidergic signaling may mediate CRH/AVP-induced suppression of tonic GnRH/gonadotropin release in female mammals. Specifically, administration of β -funaltrexamine and naloxonazine (specific MOR antagonists), but not nor-BNI (a KOR antagonist), into the POA partially restored CRH-induced LH suppression in OVX rats (Rivest et al., 1993), suggesting that MOR signaling mainly mediates the suppression. In addition, O'Byrne and colleagues (Bowe et al., 2005) demonstrated that the LV injection of naloxone restored LH pulses that were suppressed by LV administration of calcitonin gene-related peptide, another mediator of stress-induced LH suppression (Li et al., 2004), in OVX rats.

Involvement of endogenous opioid peptides in mediating pre-pubertal restraints of tonic gonadotropin-releasing hormone/gonadotropin release

It has been established that pre-pubertal restraints of GnRH/gonadotropin pulses are tightly associated with the negative feedback action of estrogen in rats and sheep (Foster and Ryan, 1979; Uenoyama et al., 2019). Endogenous opioid peptides are likely to mediate the estrogen-dependent pre-pubertal restraint of tonic GnRH/gonadotropin release in female mammals (summarized in Table 5). Ieiri et al. (1980) showed that SC administration of naloxone increased serum LH levels in pre-pubertal female rats. Ebling et al. (1989) suggested that endogenous opioidergic signaling mediates the estrogen-negative feedback action on pre-pubertal restraints of

GnRH/gonadotropin pulses in lambs because IV administration of naloxone stimulated LH pulses in ovary-intact and E2-treated pre-pubertal OVX lambs. Ebling et al. (1989) also reported that naloxone was able to further increase the frequency of LH pulses shown in OVX pre-pubertal lambs in this study. Similarly, Wood et al. (1992) showed that naloxone was able to stimulate LH pulses in OVX pre-pubertal lambs in an estrogen-dependent manner. Furthermore, Nakahara et al. (2013) showed that chronic intraperitoneal infusion of nor-BNI increased LH pulses and hence advanced puberty onset in ovary-intact female rats. Similarly, Lopez et al. (2016) showed that LV infusion of non-BNI stimulated LH pulses in pre-pubertal E2-treated OVX lambs. Taken together, these findings suggest that central opioidergic signaling, at least KOR signaling, mediates the estrogen-dependent restraint of GnRH/gonadotropin pulses during the pre-pubertal period and may serve as a key determinant of puberty onset, at least in rats and sheep. It should be noted that Lopez et al. (2016) also showed that E2 replacement failed to increase dynorphin immunoreactivity in the ARC of pre-pubertal lambs, although P4 replacement increased dynorphin immunoreactivity in the ARC of post-pubertal female sheep. Thus, non-ARC dynorphin neurons may play a key role in the pre-pubertal restraint of GnRH/gonadotropin pulses in female sheep.

It has also been established that puberty onset is associated with body growth in mammals. Indeed, growth retardation resulted in delayed puberty onset in rats and sheep (Foster and Olster, 1985; Bronson, 1986; Majarune et al., 2019). Our previous study showed that chronic food restriction (negative energy balance) during the pre-pubertal phase caused suppression of ARC *Pdyn* and *Kiss1* expression and subsequent pubertal failure in growth-retarded female rats and that *ad libitum* feeding (positive energy cues) caused an acute increase in the number of *Pdyn*- and *Kiss1*-expressing cells in the ARC, triggering puberty onset in growth-retarded female rats (Majarune et al., 2019). Similarly, Aerts et al. (2021) showed pubertal increases in *Pdyn* and *Kiss1*, but not *Tac3*, expression in the ARC of lambs. These findings suggest that dynorphin-KOR signaling and *Kiss1* (as components of KNDy neurons) serve as critical regulators of GnRH pulse generation at the onset of puberty in female mammals. The completion of KNDy mRNA and peptide expression at puberty onset is likely a prerequisite. On the other hand, there might be species differences in pubertal changes in KNDy mRNA and peptide expression: Harlow et al. (2022) demonstrated that OVX lambs with all three KNDy mRNA and peptide expression showed apparent LH pulses, whereas OVX lambs under food restriction showed suppression of *Kiss1*/kisspeptin and NKB, but not *Tac3* and *Pdyn*/dynorphin, expression and the suppression of LH pulses. It should also be noted that our and other previous studies showed that ARC *Kiss1* expression was found even in neonates and did not alter peripubertal female pigs (Ieda et al., 2014; Harlow et al., 2021). Interestingly, Ebling et al. (1990) showed that

IV administration of naloxone failed to affect pre-pubertal restraints of LH secretion in growth-retarded OVX lambs. Given that endogenous opioid peptides may mediate estrogen-dependent pre-pubertal suppression of GnRH/LH pulses in lambs, this finding suggests that inhibitory input(s), other than endogenous opioid peptides, may mainly mediate such steroid-independent inhibition of GnRH/LH secretion in pre-pubertal lambs under chronic malnutrition conditions.

Candidate populations of opioidergic neurons inhibiting tonic gonadotropin-releasing hormone/gonadotropin release

It is likely that dynorphin neurons in multiple hypothalamic nuclei—such as POA, anterior hypothalamus (AHA), and PVN—and β -endorphin neurons (also known as proopiomelanocortin neurons) located in the ARC serve as mediators of the inhibitory effect of ovarian steroids and/or stressors on GnRH/gonadotropin release in female mammals.

Foradori et al. (2005) showed that ovariectomy decreased the number of *Pdyn*-expressing neurons in the POA, AHA, and ARC compared to that in ewes at the luteal phase of the estrous cycle. The study also showed that P4 replacement restored the number of *Pdyn*-expressing cells in the POA and AHA, but not the ARC, to the level noticed in ewes at the luteal phase (Foradori et al., 2005). Our recent study showed that a systemic E2 implant that mimicked the diestrous stage significantly increased *Pdyn*-expressing cells in the PVN of OVX rats compared to OVX rats without E2 replacement (Tsuchida et al., 2020). Such a stimulatory effect of E2 on *Pdyn* mRNA expression was not found in the ARC and SON, in which dynorphin neurons were also abundantly found in female rats (Kanaya et al., 2017; Tsuchida et al., 2020). Taken together, these results suggest that POA, AHA, and/or PVN dynorphin neurons may mediate the negative feedback action of ovarian steroids on pulsatile GnRH/gonadotropin release in female mammals. It is likely that P4 directly activates *Pdyn* mRNA expression in the POA and AHA because previous studies using *in situ* hybridization or immunohistochemistry revealed that the majority of dynorphin neurons in the POA and AHA expressed nuclear progesterone receptors (PR) in ewes (Foradori et al., 2002). In addition, the majority of ARC dynorphin (KNDy) neurons expressed PR and estrogen receptor α (ER α) in ewes (Foradori et al., 2002; Franceschini et al., 2006; Smith et al., 2007) and ER α in rodents (Kinoshita et al., 2005; Smith et al., 2005; Adachi et al., 2007). It is still unclear whether PVN dynorphin neurons express ER α in rats.

Palkovits (2000) demonstrated that several stressors, such as immobilization and formalin injection, induced *Pdyn* expression in the PVN, and immobilization stress induced dynorphin-immunoreactivity in the SON of female rats. Our

previous study showed that glucoprivation induced by central and peripheral injection of 2DG increased the number of activated (*fos*-positive) dynorphin neurons in the PVN in OVX rats treated with a diestrous level of E2 (Tsuchida et al., 2020). Thus, it might be possible that PVN and/or SON dynorphin neurons likely mediate the suppression of pulsatile GnRH/gonadotropin release induced by stress or malnutrition in female mammals.

Interestingly, both fasting and glucoprivation suppressed LH pulses in female rats in an estrogen-dependent manner (Cagampang et al., 1991; Nagatani et al., 1996). Our previous studies showed that 48-h fasting induced *de novo* ER α expression in the PVN (Estacio et al., 1996) and that the local E2 implant into the PVN is needed for the fasting-induced suppression of LH in OVX rats (Nagatani et al., 1994). Thus, it is tempting to speculate that PVN dynorphin neurons may integrate ovarian steroid-negative feedback and stressor-induced signals to suppress GnRH/gonadotropin pulses, although the detailed phenotype of PVN ER α -expressing cells is currently unknown.

Whisnant et al. (1992) and Broad et al. (1993) showed that both E2 and P4 increased *Pomc* mRNA levels in the ARC of OVX ewes, suggesting that ARC β -endorphin neurons may mediate the negative feedback action of ovarian steroids on pulsatile GnRH/gonadotropin release at least in sheep. On the other hand, Wilcox and Roberts (1985) showed that E2 decreased *Pomc* mRNA levels in the ARC of OVX rats, indicating that there is a potential species difference in the regulation of *Pomc* mRNA expression by ovarian steroids. Little is known about stress-induced *Pomc* mRNA upregulation in female rodents, while fasting increased β -endorphin release from the hypothalamic explant of male rats (Mitev et al., 1993). Thus, further studies are needed to clarify how ARC β -endorphin neurons mediate the inhibitory effect of ovarian steroids and/or stressors on tonic GnRH/gonadotropin release in female rodents.

Possible action sites of endogenous opioid peptides to inhibit tonic gonadotropin-releasing hormone/gonadotropin release

Receptors for endogenous opioid peptides are widely distributed in the brain of rodents (Mansour et al., 1988, 1993, 1994, 1995a; Desjardins et al., 1990; George et al., 1994). The receptor distribution was initially examined by autoradiography (at the brain nucleus level) and later examined by *in situ* hybridization (at the cell body level) and immunohistochemistry (at the cell body and fiber levels) (Mansour et al., 1995a). The difference between the localization of binding sites (detected by autoradiography) and mRNA expression (detected by *in situ* hybridization) could be explained by receptor transportation

from the cell bodies to the axon terminals. It was reported that the MOR and KOR are widely distributed throughout the hypothalamus, whereas the DOR is scarcely distributed in the hypothalamus in rodents (Mansour et al., 1993, 1994, 1995a; George et al., 1994). Importantly, the distribution of opioid receptors are largely consistent between rodents and humans: the MOR and KOR mRNA are widely expressed, and DOR mRNA is rarely expressed in the human hypothalamus (Peckys and Landwehrmeyer, 1999).

Table 6 shows opioid receptor expression in GnRH neurons and KNDy neurons, which are considered a core component of the GnRH pulse generator, in female mammals (Lehman et al., 2010a; Okamura et al., 2013; Uenoyama et al., 2014, 2021b; Goodman et al., 2018; Moore et al., 2018; Ikegami et al., 2021). Immunohistochemical analysis revealed that the KOR is expressed in a large majority of ovine KNDy neurons and ovine and rat GnRH neurons (Lopez et al., 2016; Weems et al., 2016). In addition, *in situ* hybridization analyses revealed that KOR mRNA is expressed in the majority of ARC kisspeptin neurons in female rats (Tsuchida et al., 2020) and less than half of the ARC kisspeptin neurons in female mice (Navarro et al., 2009). Likewise, our quantitative RT-PCR analysis showed that KOR mRNA expression was detected in two of six pools of KNDy neurons (each pool consists of 10 green fluorescent protein-labeled kisspeptin cells) in female mice (Ikegami et al., 2017). On the other hand, previous *in situ* hybridization analyses revealed little MOR mRNA expression in both KNDy and GnRH neurons in female rats (Mitchell et al., 1997; Sannella and Petersen, 1997; Tsuchida et al., 2021), while MOR mRNA expression was observed in one-third of GnRH-immunoreactive cells in female guinea pigs (Zheng et al., 2005). MOR mRNA expression was found in a number of ARC non-KNDy and

POA non-GnRH neurons in female rats (Mitchell et al., 1997; Sannella and Petersen, 1997; Tsuchida et al., 2021). Taken together, these findings suggest that dynorphin-KOR signaling in the majority of KNDy and GnRH neurons may mediate the negative feedback action of ovarian steroids and stress-induced suppression of tonic GnRH/gonadotropin release in female mammals (Figure 3). In addition, inhibitory β -endorphin-MOR signaling on interneurons may somehow transmit to KNDy and GnRH neurons to suppress tonic GnRH/gonadotropin release (Figure 3). To date, studies on DOR expression in KNDy or GnRH neurons are limited, while a previous study reported no DOR mRNA expression in GnRH neurons in female rats under various steroid milieu (Sannella and Petersen, 1997).

Possible involvement of endogenous opioid peptides in gonadotropin-releasing hormone/luteinizing hormone surge generation

Previous studies suggest that a transient decrease in the endogenous opioid tone contributes to the initiation of the preovulatory LH surge in female mammals (Gabriel et al., 1983; Allen and Kalra, 1986; Rossmannith et al., 1988; Walsh and Clarke, 1996; Smith and Gallo, 1997). Concretely, IV or SC administration of naloxone advanced the onset of LH surge induction and increased the amplitude of LH surge in women with normal cycles (Rossmannith et al., 1988) and in proestrous or estradiol benzoate (EB)-treated OVX rats (Gabriel et al., 1983; Allen and Kalra, 1986). Furthermore, Smith and Gallo (1997)

TABLE 6 Expression of opioid receptor mRNAs in gonadotropin-releasing hormone (GnRH) and KNDy neurons in female mammals.

Neurons	Receptors	Expression rates	Species	Gonadal states	Methods	References
GnRH	μ -	0%	Rat	Proestrus	ISH	Mitchell et al., 1997
	μ -	0%	Rat	Intact/OVX/OVX + E2/OVX + E2 + P4	ISH	Sannella and Petersen, 1997
	μ -	33.3%	Guinea pig	OVX	ISH + IHC ¹	Zheng et al., 2005
	μ -	0%	Rat	OVX + E2	ISH	Tsuchida et al., 2021
	δ -	0%	Rat	Intact/OVX/OVX + E2/OVX + E2 + P4	ISH	Sannella and Petersen, 1997
	κ -	0%	Rat	Proestrus	ISH	Mitchell et al., 1997
	κ -	0%	Rat	Intact/OVX/OVX + E2/OVX + E2 + P4	ISH	Sannella and Petersen, 1997
	κ -	95.4%	Sheep	luteal phase	IHC	Weems et al., 2016
	κ -	95.4%	Rat	OVX + E2 + P4	IHC	Weems et al., 2016
KNDy	μ -	0.4%	Rat	OVX + low E2	ISH	Tsuchida et al., 2021
	κ -	20%	Mouse	OVX/OVX + E2	ISH	Navarro et al., 2009
	κ -	33%	Mouse	OVX	qRT-PCR ²	Ikegami et al., 2017
	κ -	97.8%	Sheep	luteal phase	IHC	Weems et al., 2016
	κ -	62%	Rat	OVX + low E2	ISH	Tsuchida et al., 2020

ISH, *in situ* hybridization; IHC, immunohistochemistry.

¹GnRH neurons were detected by IHC.

²*Oprk1* (coding KOR) expression in pooled KNDy cells was analyzed by qRT-PCR.

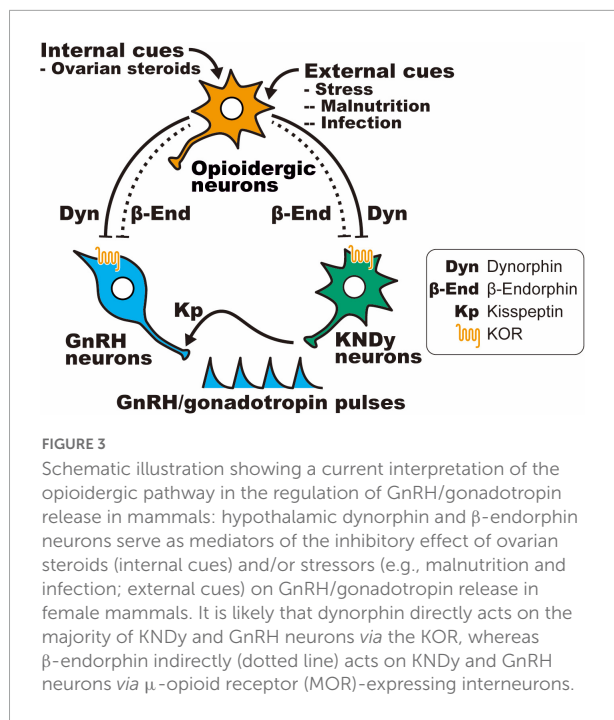


FIGURE 3
Schematic illustration showing a current interpretation of the opioidergic pathway in the regulation of GnRH/gonadotropin release in mammals: hypothalamic dynorphin and β -endorphin neurons serve as mediators of the inhibitory effect of ovarian steroids (internal cues) and/or stressors (e.g., malnutrition and infection; external cues) on GnRH/gonadotropin release in female mammals. It is likely that dynorphin directly acts on the majority of KNDy and GnRH neurons via the KOR, whereas β -endorphin indirectly (dotted line) acts on KNDy and GnRH neurons via μ -opioid receptor (MOR)-expressing interneurons.

showed that nor-BNI infusion into the medial POA advanced the onset of LH surge in proestrous female rats. In addition, Walsh and Clarke (1996) showed that an MOR agonist, but not KOR and DOR agonists, delayed the onset of the EB-induced LH surge in OVX ewes. These findings suggest that endogenous opioid peptides may exert an inhibitory influence on GnRH/LH surge generation.

It is well accepted that another population of hypothalamic kisspeptin neurons, which are located in the anteroventral periventricular nucleus (AVPV) in rodents (Smith et al., 2005, 2006; Adachi et al., 2007; Clarkson et al., 2008) and the POA in several mammalian species, including macaque monkeys (Smith et al., 2010; Watanabe et al., 2014), sheep (Smith et al., 2009), goats (Matsuda et al., 2015), cattle (Hassaneen et al., 2016), and musk shrews (Inoue et al., 2011), as well as in the periventricular nucleus in pigs (Tomikawa et al., 2010), have been considered to serve as a target of estrogen-positive feedback action to induce GnRH surge in female mammals (see review article for details, Uenoyama et al., 2021a; Goodman et al., 2022; Tsukamura, 2022). Interestingly, previous studies showed coexpression of *Penk*/Met-enkephalin and *Pdyn* in the majority of AVPV kisspeptin neurons in female mice (Porteous et al., 2011; Stephens and Kauffman, 2021). To the best of our knowledge, little is known about the physiological roles of Met-enkephalin and dynorphin in AVPV kisspeptin neurons, although these findings tempt us to speculate that Met-enkephalin and/or dynorphin may have a role as a regulatory signal for LH surge generation in an autocrine/paracrine fashion in mice. Stephens and Kauffman (2021) showed that *Pdyn* expression was higher in OVX mice than E2-treated OVX mice,

suggesting that dynorphin may suppress the onset of LH surge in an autocrine/paracrine fashion. Further studies are needed to uncover the precise mechanism by which endogenous opioid peptides regulate LH surge generation in female mammals.

Conclusion and perspectives

Based on the findings currently available, we can envisage that hypothalamic opioidergic neurons play several important roles in the brain mechanism, regulating reproduction in female mammals. In particular, ARC dynorphin neurons, which are now known as KNDy neurons because of the coexpression of NKB and kisspeptin, are recognized as the GnRH pulse generator that governs female reproduction by controlling tonic GnRH/gonadotropin release throughout the estrus/menstrual cycles. In addition, dynorphin neurons located in several hypothalamic nuclei, such as the POA, AHA, and/or PVN, are likely to serve as mediators of ovarian steroid-negative feedback action on tonic GnRH/gonadotropin release by suppressing KNDy and/or GnRH neuronal activity via the KOR expressed in KNDy and/or GnRH neurons in female mammals. It is also postulated that ARC β -endorphin neurons may also mediate ovarian steroid-negative feedback action and suppress KNDy and GnRH neuronal activity via MOR-positive interneurons. Furthermore, hypothalamic opioidergic neurons are also likely to serve as mediators of external adverse cues, such as malnutrition and infection, and suppress tonic GnRH/gonadotropin release under stressful conditions. To date, findings have mainly been accumulated for MOR and KOR signaling, and little is known about whether DOR signaling serves as a mediator of ovarian steroid-negative feedback action and/or stress-induced suppression of tonic GnRH/gonadotropin release in female mammals. To uncover the precise roles of hypothalamic opioidergic neurons in mammalian reproduction as a whole, further studies are needed to clarify precise opioidergic neural pathways that control KNDy and GnRH neuronal activity in female mammals.

Author contributions

YU and HTk collected the information and wrote the manuscript. HTc, MN, and NI critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships

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that could be construed as a potential conflict of interest.

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