



ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(7): 4288-4301
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www.thepharmajournal.com
Received: 08-05-2022
Accepted: 12-06-2022

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Application of GnRH modulators in controlling reproductive cycle in farm animals: A review

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Abstract

The hypothalamic-pituitary-gonadal axis (HPG axis) alludes to the connection between the hypothalamus, pituitary gland, and gonads. A number of intrinsic and extrinsic factors are integrated in the HPG axis that regulates the core functions of the reproductive system. A wide variety of GnRH modulators are already in practice but desired level of response is not achieved mostly due to their action at the downstream of HPG axis. In the recent past, novel neuro-peptide molecules like kisspeptin, phoenixin, etc. have been identified. The localization pattern of these molecules suggests a key role in the upstream of hypothalamic nuclei to regulate the reproductive processes. In farm animals, Kisspeptin plays a wide variety of actions, mainly regulating GnRH-mediated gonadotrophin release during onset of puberty, induction of estrus in the breeding season and extra-hypothalamic regulation of ovarian functions. Natural Kisspeptin molecules have limited therapeutic applications owing to their shorter half-life leading to the development of a series of synthetic Kisspeptin analogues. A few studies on kisspeptin agonists, namely Compound-6 and Compound-17 showed better performance in ovine, caprine and laboratory animal model over the natural kisspeptin. Recently, Kisspeptin antagonists (P²³⁴) have also been used in *in vivo* studies showing promising effect in controlling GnRH mediated LH surge. Emerging evidence suggests use of Kp and other neuropeptide analogues in the therapeutic protocols of controlled reproduction programs in several farm animal species. Thus, there is a need to put more efforts in the optimization of promising GnRH modulators for use in farm animals in order to augment reproductive efficiency.

Keywords: Kisspeptin, hypothalamus, cyclicity, GnRH, farm animals

1. Introduction

Reproduction is the most important phenomenon to ensure the survival of a species on the earth. Brain control of mammalian reproduction involved integration of number of factors which include the gonadal status of the animal, age and body condition score of the animal, pheromones, stressors, various body rhythms (ultradian, circadian, infradian and circannual rhythm) and seasonality in some species. In females, these factors show their impact on the ability to produce fertile gametes, completion of fertilization process, attain pregnancy, carrying the fetus to term and nurse the offsprings. Reproduction in males is also influenced by many of these factors. The mechanism of reproduction is under the control of Hypothalamus-Pituitary-Gonadal axis. As functioning of Hypothalamus, Pituitary gland and gonads are inter-related and often act as a single unit, so commonly referred as Hypothalamus-Pituitary-Gonadal axis (HPG axis). Hypothalamus, being the controller of axis, regulated by various factors, produces and secretes the GnRH hormone which reaches the anterior pituitary gland via the Hypothalamo-hypophyseal portal system and stimulates gonadotroph cells leading to production of gonadotrophin hormones i.e. Follicle Stimulating Hormone and Luteinizing Hormone. GnRH is found to be released in two fashions i.e. pulsatile pattern and surge fashion of release. Pulsatile release of GnRH is controlled by arcuate nucleus while the surge release is under the control of both arcuate and pre-optic nuclei.

Gonadotropins act on ovary to maintain the cyclicity and the production of ovarian steroids (Estrogen, Progesterone and Androgen) and other hormones like inhibin and activin. Ovarian steroids control the release of gonadotrophins by the feedback mechanism. Estrogen (E2) and Progesterone (P4) shows negative feedback action on the arcuate nucleus while E2 gives positive feedback action to both arcuate and pre-optic area prior to the ovulation resulting in LH surge. Recent studies suggest progesterone also playing some part in positive feedback mechanism.

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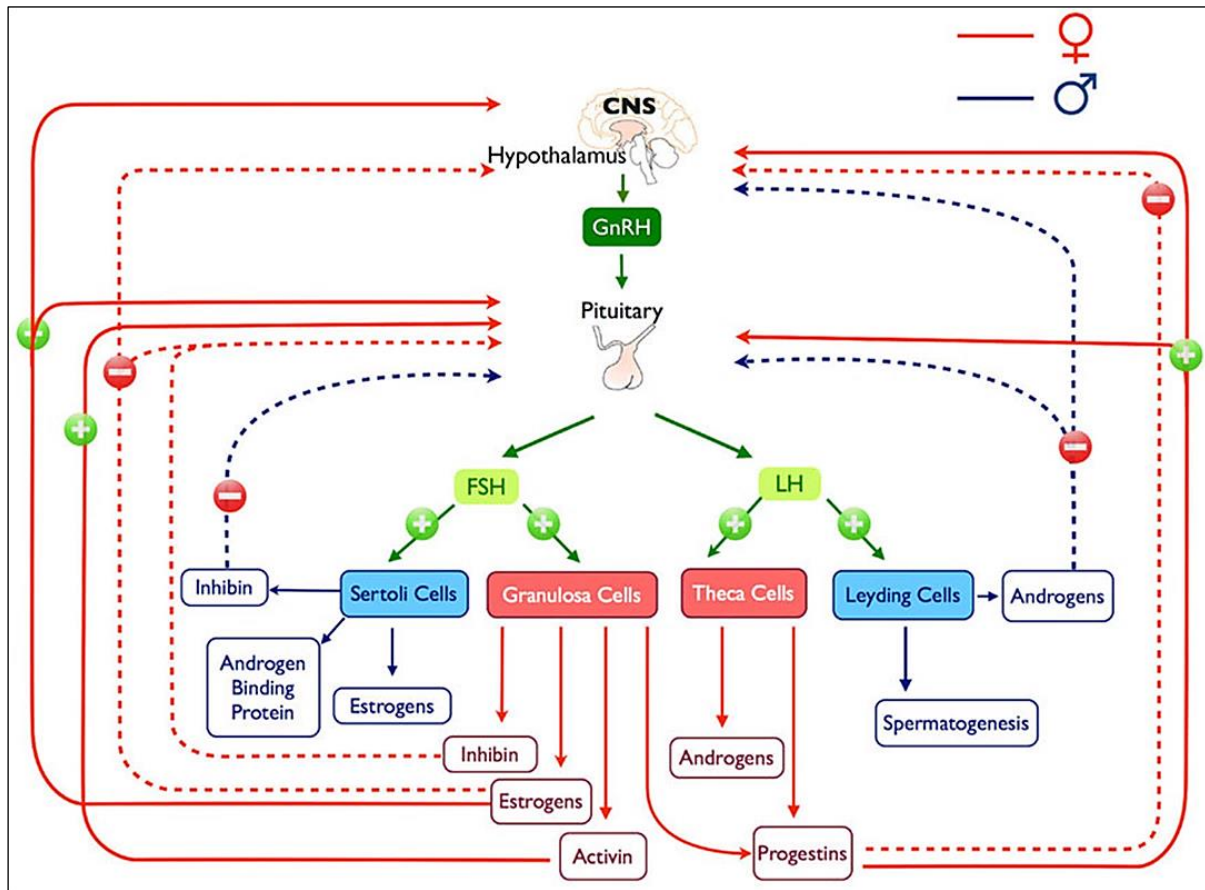


Fig 1: Hypothalamus-Pituitary-Gonadal axis

2. Incidence of reproductive disorders

Proper correlation of HPG axis is must to keep the process of reproduction going on and any deviation leads to infertility in animals. Infertility can be due to several reasons but anestrus, repeat breeding and delayed sexual maturity are found to be the most common non-infectious causes. Economic impact of infertility is significant as it leads to increased calving intervals and therefore results in reduced calf crop, decreased life-time milk yield, increased culling rates, etc. 10-30% of lactations are affected by infertility and reproductive disorder

in developed countries (Erb and Martin, 1980) [32].

Anestrus, being the functional form of infertility, is simply the failure of exhibition of estrus by the female animal. It can be due to physiological (pregnancy, prepubertal or postpartum) or pathological (fetal mummification, pyometra, etc.) reasons. Anovulatory estrus is the true form of anestrus while the ovulatory anestrus can be due to improper observation/ missed heat, persistent corpus luteum and subestrus/silent estrus. Silent estrus is a more common disorder of buffaloes in summer months.

Table 1: Incidence of Anestrus condition in bovines in India

State	Species	Anestrus (%)	References
Andhra Pradesh	Cattle	49.70	Rao, 1993 [101]
	Buffalo	30.76-50	Rao and Sreemannarayanan, 1982 [102]
Gujarat	Cattle	24.73	Patel <i>et al.</i> , 2007 [95]
	Buffalo	20.84-45.97	Modi <i>et al.</i> , 2011 [85]
	Cattle	53.15	Pandit, 2004 [94]
Maharashtra	Buffalo	29.12-60.83	Pandit, 2004; Kumar <i>et al.</i> , 2013 [94, 64]
	Cattle	2.13-45.97	Narladkar <i>et al.</i> , 1994 [89]
Tamil Nadu	Buffalo	29.5-41.4	Bharkad and Markandeya, 2003 [9]
	Cattle	16.6	Selvaraju <i>et al.</i> , 2005
Punjab	Buffalo	9.09	Selvaraju <i>et al.</i> , 2005
	Cattle	43-67.11	Singh <i>et al.</i> , 2003 [112]
Bihar	Buffalo	38.98-55.5	Singh <i>et al.</i> , 2006 [113]
	Cattle	39.01	Singh, 1981 [111]
Kerela	Cattle	65	Kutty and Ramachandran 2003 [66]
Kashmir	Cattle	27.52-31	Bhattacharyya and Buchoo, 2008 [10]
Uttar Pradesh	Cattle	3.32	Verma S K <i>et al.</i> , 2018 [124]
	Buffalo	14.69-45.20	Luktuke <i>et al.</i> , 1973 [73]
Karnataka	Buffalo	56	Hussain, 1984 [53]
Haryana	Bovine	28.66	Meena M S <i>et al.</i> , 2009 [75]
Meghalaya	Cattle	10.76	Khan M H <i>et al.</i> , 2016 [60]
Orissa	Cattle	37.39	Harichandan P.P. <i>et al.</i> , 2018 [49]

3. Existing modulators of HPG axis in practice

Table 2: Summary of molecules showing effect on reproductive axis

Molecule	Mechanism	Effect
Buseralin acetate	GnRH-R agonist	Stimulate LH and FSH release
Acyline	GnRH-R antagonist	Inhibit release of LH and FSH
hCG	LH-R agonist	Simulates the actions of endogenous LH
pFSH	FSH receptors	Performs all actions of endogenous FSH
eCG	FSH receptors LH receptors	Has predominantly FSH like activity with somewhat LH like activity
Progestogens	Progesterone receptor agonists	Inhibit LH release
Epostane	Blocks β -hydroxy steroid dehydrogenase isomerase enzyme	Prevents synthesis of progesterone
Estradiol	Estrogen receptor agonists	Negative feedback on tonic GnRH release Positive feedback on surge release of GnRH
Tamoxifen citrate	Estrogen-R antagonist	Blocks the effect of estrogen
Dinoprost	Natural PGF 2α	Lyse corpus luteum and removes P4 dominance
Cloprostenol	Synthetic PGF 2α	Lyse corpus luteum and removes P4 dominance
Melatonin	Indirect action via dopaminergic neurons	Transition from non-breeding to breeding season i.e. onset of cyclicity
Noradrenaline	norAdr-r in POA and ARC	Stimulatory role on LH surge release Inhibitory effect on tonic LH release
Growth hormone	Growth hormone receptors	Advances pubertal age
GABA	POA	Inhibits GnRH/ LH secretion
Leptin	Leptin receptors	Stimulate LH release during pubertal escape from negative feedback of estrogen.

All of the above molecules act at the peripheral part of the reproductive axis and either overstimulation or shows over-inhibitory effect on endogenous secretion of reproductive

hormones leading to significant side effects. There is much more variability in their effects.

Table 3: Summary of responses of existing GnRH modulators

Compound	Animal model	Response (Conception rate)	Reference
Buseralin acetate	Cattle	28.1%	Stevenson <i>et al.</i> 2007 [55]
Ovsynch protocol	Buffalo (anestrus)	37.5%	Kumar <i>et al.</i> 2016 [67]
hCG	Cattle	33.6%	Stevenson <i>et al.</i> 2007 [55]
Cloprostenol	Cattle	65.2% with 59.4% ODR	Baryczka <i>et al.</i> 2018 [11]
CIDR synchronized	Cattle	45.5% with 79.5% ODR	Romano <i>et al.</i> 2013 [58]
Melatonin	Buffalo	32.4% with 90% ODR	Kumar <i>et al.</i> 2018

These disadvantages limit their use for modulation of estrous cycle and necessitating the discovery of new and more potent modulators.

4. New players in reproductive function

With the advancement in science, scientists came forward with many new molecules acting superiorly to GnRH and showing significant effect on HPO axis. In Sheep, the neurons producing GnRH receive relatively little synaptic inputs with respect to other neurons in the Hypothalamus (Decourt *et al.* 2014, Lehman *et al.* 1988, Magee *et al.* 2009, McGrath *et al.* 2016) [69, 77, 82]. Wide array of different neurons control GnRH activity but one particular set of neurons come to prominence-those producing Kisspeptin.

4.1 Kisspeptin

In 1996, KISS1 gene was discovered as a metastasis-suppressor gene in Hershey, Pennsylvania (Lee *et al.*, 1996) [68]. KISS1 gene encodes a 145 amino-acid polypeptide, later which gets cleaved into several isoforms (all sharing a common C-terminal decapeptide sequence)-collectively called kisspeptins (Kotani M. *et al.*, 2001) [63]. The biologically active peptides are named as per the number of amino-acid they are having i.e. (longest forms: Kp54 in human, Kp53 in sheep or Kp52 in rodents and smaller forms: Kp16, Kp14, Kp13 and Kp10) (Decourt *et al.*, 2018) [19]. Kisspeptin is a family of neuropeptides that acts as high-level mediators of

the HPG axis acting upstream to level of GnRH. These neuropeptides activate the orphan G-protein coupled receptor, which later termed as KISS1R (Muir *et al.*, 2001) [87]. Earlier studies revolved around the use of kisspeptin as metastasis suppressor molecule for melanoma and breast cancer cell lines. Its role in reproductive endocrinology was hypothesized after its discovery in human placental extracts and subsequent identification of its expression in hypothalamus, pituitary and gonads (Bilban *et al.*, 2004 and Seminara *et al.*, 2008) [110]. Kp10 sequence is relatively similar among species with some variations suggesting a conserved physiological function (Oakley *et al.*, 2009) [92]. Kp54 and Kp10 shows similar potency and biological activity *in vitro*, however susceptibility of Kp10 to enzymatic cleavage limits its potency *in vivo*. The half-life of Kp10 in healthy men and women was 3.8-4.1 min and for Kp54 it was 27.6 min (Dhillon WS *et al.*, 2005 and George JT *et al.*, 2011) [27, 39].

Human Kp-10 Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH₂ (Albers-Wolthers *et al.* 2017) [2].

Canine Kp-10 Tyr-Asn-Trp-Asn-Val-Phe-Gly-Leu-Arg-Tyr-NH₂ (Albers-Wolthers *et al.* 2017) [2].

Bovine Kp-10 Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr-NH₂ (Pottapenjera *et al.* 2018) [98].

4.1.1 Location of kisspeptin neurons

The largest population of kisspeptin neurons is located in the arcuate nucleus of hypothalamus and especially in the more

caudal region of nucleus extending around pre-mammillary body. This is seen in sheep (Estrada *et al.* 2006, Franceschini *et al.* 2006, Goodman *et al.* 2007) [33, 37, 43], goat (Okamura *et al.* 2017), cattle (Hassaneen *et al.* 2016, Tanco *et al.* 2016) [50], pigs (Tomikawa *et al.* 2010) [122] and horses (Decourt *et al.* 2008, Magee *et al.* 2009) [26, 77]. Second largest population, being much smaller in number, is in medial pre-optic area of sheep, goats and cattle (Estrada *et al.* 2006, Franceschini *et al.* 2006, Goodman *et al.* 2007, Matsuda *et al.* 2015, Hassaneen *et al.* 2016, Tanco *et al.* 2016) [33, 37, 43, 79, 50]. Pigs have kisspeptin neurons in the periventricular nucleus instead of pre-optic area (Tomikawa *et al.* 2010) [122]. Interestingly, horses do not contain any kisspeptin neuron in pre-optic area at all (McGrath 2015) [81].

4.1.2 KNDy neurons

Neurokinin-B and Dynorphin receptors are co-expressed in nearly all the kisspeptin neurons located in arcuate nucleus (Goodman *et al.* 2007, Wakabayashi *et al.* 2010, Hassaneen *et*

al. 2016) [43, 125, 50], therefore, commonly referred as KNDy neurons. KNDy neurons are not expressed in other locations of kisspeptin neurons. In goats, extensive communication has been shown between right and left-hand sides of nucleus by tract tracing studies (Wakabayashi *et al.* 2013) [126], mainly via Neurokinin-B. Majority of KNDy neurons express NK3 receptors (receptor for Neurokinin-B) in sheep (Amstalden *et al.* 2010) [3] and goats (Wakabayashi *et al.* 2013) [126] while kappa opioid receptors are expressed in about 90% of KNDy neurons in sheep (Weems *et al.* 2016) [128]. But contrastingly, kisspeptin receptors are not expressed in these neurons (Smith *et al.*, 2011) [117] indicating that communication among them is via Neurokinin-B and Dynorphin only not by the kisspeptin molecule. Recent studies have shown that Neurokinin-B transcripts are upregulated in the POA of buffaloes in follicular and mid-luteal stages while Dynorphin transcripts are downregulated in the POA in early luteal phases (Mishra *et al.* 2019) [38].

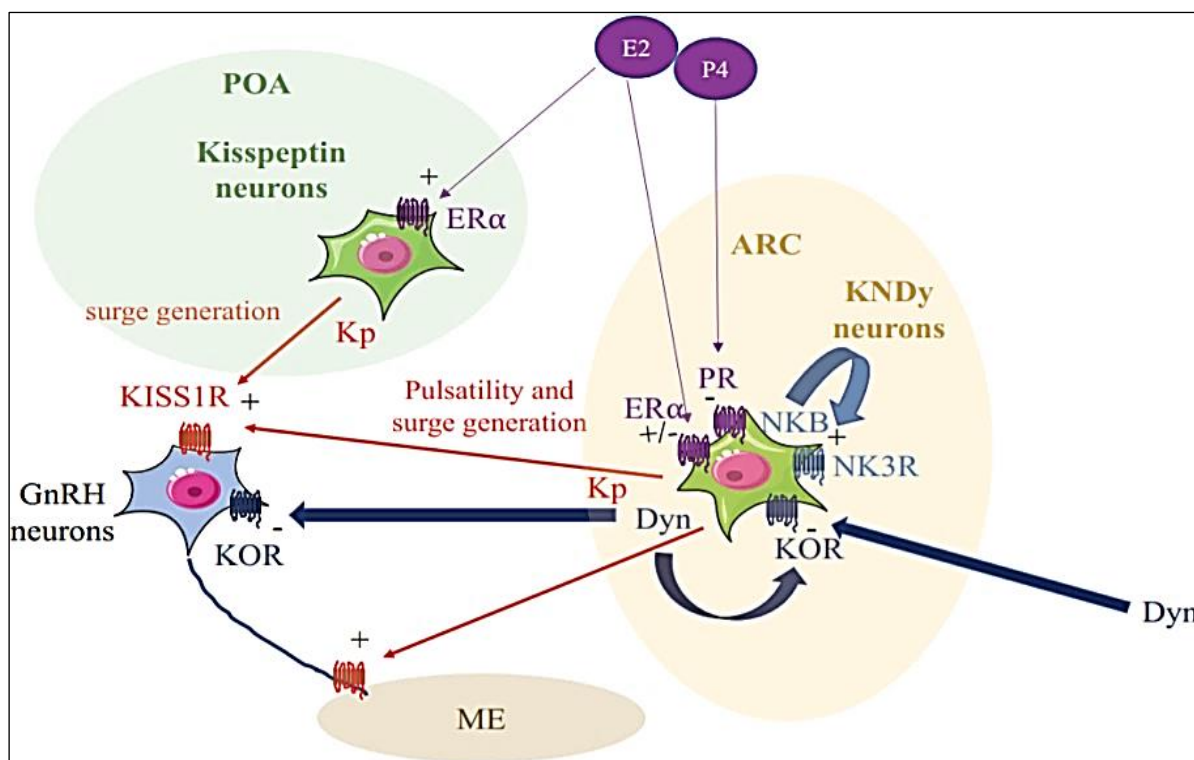


Fig 2: Diagrammatic representation of Kp (Kisspeptin) and KNDy (Kisspeptin, Neurokinin B, Dynorphin) neurons regulation in adult ewes

Abbreviations: POA (Pre-Optic Area), ARC (Arcuate nucleus), ME (Median Eminence), GnRH (Gonadotropin Releasing Hormone), NKB (Neurokinin B), Dyn (Dynorphin), E2 (17 β Estradiol), P4 (progesterone), KISS1R (Kp Receptor), NK3R (NKB Receptor), KOR (Dyn Receptor), ERα (Estrogen Receptor α), PR (Progesteron Receptor).

The kisspeptin fibres located in median eminence seems to be of KNDy origin indicating their origin in arcuate nucleus (Smith *et al.*, 2011) [117]. Similarly, most inputs to kisspeptin neurons of preoptic area are also from KNDy neurons (Smith *et al.*, 2011; Merkley *et al.*, 2015) [117, 83]. Sex differences in terms of kisspeptin neurons have been observed in sheep and was firstly observed for Neurokinin-B (Goubillon *et al.*, 2000) [46], long before the discovery of KNDy neurons where rams showed fewer immunoreactivity than the ewes. Later, it was confirmed for KNDy neurons where rams showed nearly half

the neurons in arcuate nucleus than the ewes (Cheng *et al.*, 2010) [22]. This difference is manifest primarily in the most caudal parts of the arcuate nucleus (Goubillon *et al.* 2000, Cheng *et al.* 2010) [46, 22], with little difference in cell numbers in the rostral arcuate. Similarly, significantly higher numbers of kisspeptin-ir neurons were found in the preoptic area of ewes compared with rams (Cheng *et al.* 2010) [22]. Sex difference in neuronal population is most probably due to the organizational action of testosterone (Cernea *et al.* 2015) [20]. Prenatal treatment of ewe lambs with testosterone results in smaller KNDy cells and number of synaptic inputs are also reduced especially KNDy to KNDy cells along with number of inputs to GnRH neurons. However, number of KNDy cells remained same showing testosterone action during embryonic development in not the sole reason for sex difference in the population of KNDy cells (Cheng *et al.*, 2010) [22].

4.2 Factors regulating Kisspeptin neurons

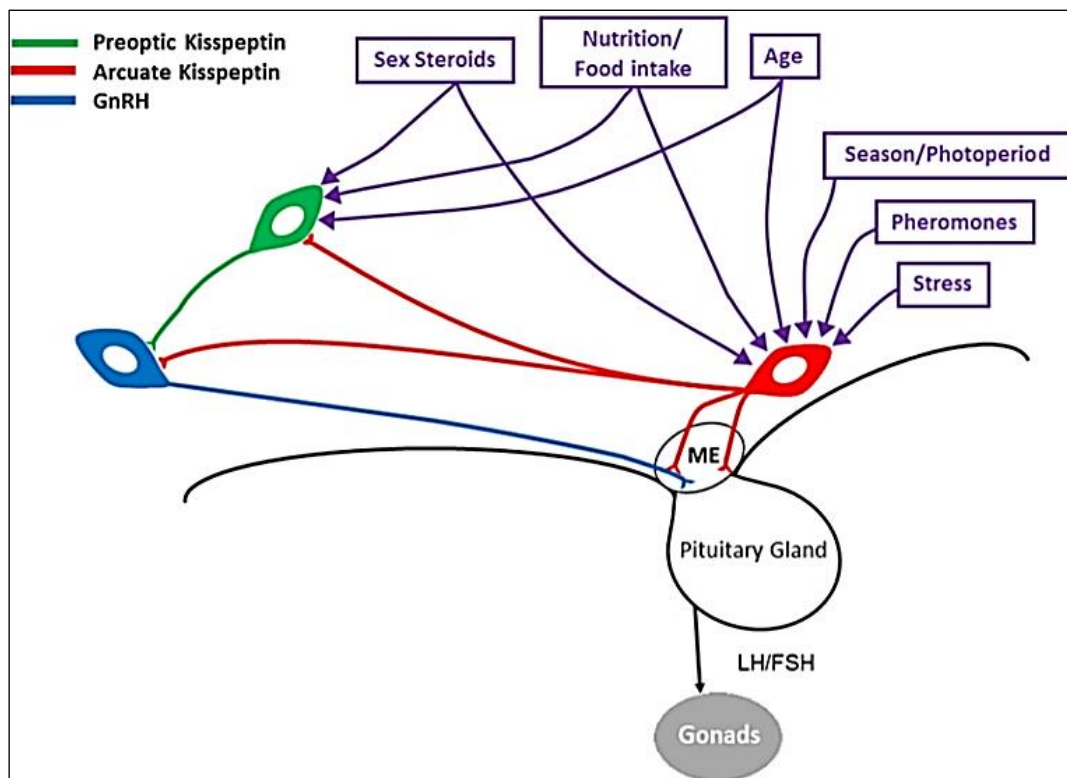


Fig 3: Model for the integration of factors from the internal and external environment in the brain control of reproduction in domestic animals

4.2.1 Role of steroid hormone in GnRH regulation

Nearly half of preoptic kisspeptin neurons express Estrogen receptor- α and almost all of the arcuate kisspeptin neurons express Estrogen receptor- α in ewes (Goubillon *et al.* 2000, Franceschini *et al.* 2006) [46, 37], progesterone receptors (Foradori *et al.* 2002, Dufourny *et al.* 2005, Smith *et al.* 2007) [35, 29, 115] and androgen receptors (Rose 2017) [104]. Studies using sex steroids as intracranial implants in sheep (Blache *et al.* 1997, Scott *et al.* 1997, Caraty *et al.* 1998) [13, 107, 16] indicate that in males and females, the arcuate nucleus, and to a lesser extent the preoptic area are key sites for the actions of sex steroids in the hypothalamus. Thus, the sex steroids may act directly on kisspeptin neurons.

Estradiol

Estrogen treatment in ovariectomized ewes results in reduction in neurokinin B mRNA levels (Pillon *et al.* 2003) [97]. Later studies in sheep showed regulation of kisspeptin neurons by estrogen in an inhibitory manner at lower doses while higher doses stimulate them.

Negative feedback: Increased mRNA expression of KISS1 gene in the arcuate nucleus (but not preoptic) (Smith *et al.* 2007) [115] and higher levels of Fos expression in KNDy neurons are observed in ovariectomized ewes than intact ones. These changes are blocked by chronic administration of estrogen (Merkley *et al.* 2012) [84] indicating negative feedback action of estrogen on arcuate nucleus neurons. Within KNDy neurons, ovariectomy stimulates kisspeptin and Neurokinin B (Smith *et al.* 2007, Nestor *et al.* 2012) [115, 90] along-with inhibiting dynorphin (Foradori *et al.* 2006) [34] expression. Based on this evidence, it would seem likely that sex steroids must change the degree of co-localisation of kisspeptin, neurokinin B and dynorphin (and glutamate) within KNDy neurons (Goodman *et al.* 2013) [41], but direct

testing of this hypothesis has not been done.

Positive feedback: An elevation in the expression of KISS1 mRNA in arcuate nucleus is noticed during the late follicular phases at times when higher levels of estrogen in circulation are there. Similar increase in neurokinin B mRNA levels in mid arcuate area was noticed (Li *et al.* 2015) [70]. Administration of estradiol benzoate in higher doses (i.e. sufficient to induce LH surge) to ovariectomized ewes resulted in increased expression of Fos in arcuate nucleus indicating neuronal activation at that time (Smith *et al.*, 2009a) [116]. In addition, the number of synaptic inputs to kisspeptin neurons in the arcuate nucleus was higher in estrous ewes in comparison with ewes in the luteal phase (Merkley *et al.* 2015) [83]. Increased Fos and KISS1 mRNA expression are noticed in the kisspeptin neurons of preoptic area at the onset of preovulatory LH surge in ewes (Hoffman *et al.* 2011, Smith *et al.* 2009a) [52, 116]. Interestingly, in pigs, there is reduction in number of kisspeptin-ir cells in arcuate nucleus after a high dose of estrogen/estrogen peak while an increase in kisspeptin cells is noticed in periventricular region (Tomikawa *et al.*, 2010) [122]. In goats, there is increase in expression of Fos in preoptic area but no change in arcuate nucleus (Matsuda *et al.*, 2015) [79]. This suggests that the regulation of kisspeptin by estrogen in the pig and goat is more similar to that of the rat and mouse than in the sheep. Presence of activated progesterone receptors is necessary for the positive feedback action of increasing concentrations of estradiol at the time of preovulatory surge (Mishra *et al.* 2019) [38].

Progesterone

Progesterone treated ovariectomized ewes show moderately lower levels of KISS1 mRNA but effect was much smaller w.r.t estrogen treatment (Smith *et al.*, 2007) [115]. Progesterone

treatment showed no effect in the expression of preprodynorphin mRNA levels in ovariectomized ewes (Foradori *et al.*, 2005) [36]. Estrogen is needed to upregulate progesterone receptors (to allow P4 to have much of an effect) in arcuate nucleus (Scott *et al.*, 2000a) [108]. Cows shows differences in kisspeptin cell numbers in the late follicular phase and luteal phase in preoptic area (Hassaneen *et al.* 2016) [50] and in arcuate area (Tanco *et al.* 2016). Study by Hassaneen *et al.* 2016 [50] involved differences in both circulating estrogen and progesterone levels led to consideration by authors as differences most likely due to varied estrogen levels. But in the study by Tanco *et al.* 2016 circulating estrogen levels were similar in both groups and significant differences in P4 levels indicating the major role of progesterone in inhibiting kisspeptin expression in arcuate nucleus.

Testosterone

Very few kisspeptin-ir cells could be detected in intact rams (Nestor *et al.* 2012, Rose 2017) [90, 104] or bucks (Matsuyama *et al.* 2011) [80], but large numbers of kisspeptin-ir cells were observed in the caudal arcuate nucleus in males that had been castrated (Matsuyama *et al.* 2011, Nestor *et al.* 2012, Rose 2017) [80, 90, 104] indicating testosterone exerting a very strong inhibitory action on kisspeptin neurons in male sheep.

4.2.2 Season/Photoperiod

Kisspeptin neurons are regulated by season/photoperiod when they observed higher number of KISS1 mRNA containing cells in arcuate nucleus of sheep during breeding season than the non-breeding season while no such difference was observed in preoptic area (Smith *et al.* 2007) [115]. Similar result was also observed in Abadeh goat does (Jafarzadeh Shirazi *et al.* 2014) [56] and mare (McGrath 2015) [81]. These changes in kisspeptin cell numbers coincide with changes in Neurokinin-B cell numbers but not dynorphin in estrogen implanted ovariectomized ewes (Weems *et al.* 2017) [127] indicating a seasonal change in the degree of co-localization in KNDy neurons.

Transfer of ewes from long day photoperiod to artificial short-day photoperiod result in higher number of kisspeptin neurons in the arcuate nucleus (Chalivoix *et al.* 2010) [21]. Melatonin is said to act indirectly on kisspeptin neurons as melatonin receptors are not expressed on kisspeptin neurons (Li *et al.* 2011) [71] but express dopamine D2 receptors (Goodman *et al.* 2012) [44]. In sheep, dopamine neurons present in the retrochiasmatic area of the hypothalamus exert inhibitory action on GnRH secretion during anestrus but not during breeding season (Goodman *et al.* 2010) [42]. D2 receptor expression on kisspeptin neurons is seasonally regulated i.e. twice the KNDy neurons expressing this receptor in non-breeding season (80%) than the breeding season (40%) (Goodman *et al.* 2012) [44].

4.2.3 Nutrition

Neuronal systems, associated with metabolic status of the individual like Neuropeptide Y, orexin and proopiomelanocortin (Norgren & Lehman 1989) [91] gives direct inputs to GnRH neurons allows direct control over GnRH release and influence over reproduction. Lean sheep showed lower levels of KISS1 mRNA in both preoptic and arcuate nucleus W.R.T. control fed ewes and intracerebroventricular injection of leptin reverses its effect (Backholer *et al.* 2010) [7]. Leptin receptors are expressed in

virtually all preoptic and arcuate kisspeptin neurons suggesting direct effect of leptin on kisspeptin (Backholer *et al.* 2010) [7]. In ewe, arcuate kisspeptin neurons receive direct inputs from NPY and POMC (Backholer *et al.* 2010) [7]. High food intake in heifers increased kisspeptin neuronal population receiving apposition from fibres that were immunoreactive for α MSH (POMC product) (Cardoso *et al.* 2015) [18].

4.2.4 Stress

Some stressors like transport, heat, isolation/restraint, hypoglycaemia, LPS injection and barking dog can exert significant inhibitory action on reproduction while some acute stresses can stimulate, especially in pigs (Einarsson *et al.* 2008) [30]. Part of the inhibitory action is mediated by cortisol acting via type 2 glucocorticoid receptors (expressed by KNDy neurons) increase levels of pre-prodynorphin mRNA in arcuate nucleus of ewes (Ralph *et al.* 2016) [100].

4.2.5 Pheromones

In ewes and does (especially late anestrus), the ram effect resulted in elevation in KISS1 mRNA levels and fos activity in rostral and mid-arcuate regions (but not in caudal arcuate) inducing a rapid increase in GnRH/LH pulse frequency and therefore ovulation (De Bond *et al.* 2013) [24]. Hair from bucks (having high levels of pheromones) induced an increase in bursts of electrical activity in KNDy neurons and were suppressed by NK3 receptor antagonist (SB22200) (Sakamoto *et al.* 2013) [126]. "Male effect" is seen in pigs (Kirkwood *et al.* 1981) [61] but no published data about role of kisspeptin in its action.

4.3 Role of kisspeptin in reproductive physiology

4.3.1 Pituitary

Kisspeptins are identified in the ovine hypophyseal portal blood (Smith *et al.* 2008) [114] leading to hypothesis that kisspeptin may act at the level of pituitary to directly induce LH secretion from the gonadotroph cells. GnRH antagonist inhibits the typical kisspeptin-induced increase in LH (Irwig, 2004) [54], indicating that the primary actions of kisspeptin on gonadotropin secretion occur upstream of the pituitary.

4.3.2 Gender differentiation

Kisspeptin system is apparently critical for brain gender differentiation, acting via regulation of postnatal testosterone secretion. Anatomical differences have been reported as females having greater kisspeptin neuronal population in some species like mice, ewe and doe (Nestor *et al.* 2012, Rose 2017 and Matsuyama *et al.* 2011) [90, 104, 80].

4.3.3 Onset of puberty

Four major components that are likely to participate in Hypothalamic KISS1 system control over puberty onset are:- (Bhalakiya *et al.* 2019) [8]

1. Increase in endogenous kisspeptin tone driving HPO axis to its full activation.
2. Increased sensitivity of GnRH/LH responses to stimulation by kisspeptin.
3. Increased efficiency of GPR54 signalling.
4. Increased number of kisspeptin neurons at AVPV/POA/PVN and/or arcuate nuclei as per the species as well as increase in number of projections to GnRH neurons.

4.4 Kisspeptin agonists

Kp10

KP-10 shows promising results in the *in vitro* tests but effect is of very shorter duration *in vivo* due to its shorter half-life and requiring multiple injections to get the desired effect. Metabolic degradation and quick renal clearance limits the use of natural kisspeptins. Also, desired effects can only be achieved only by i/v or intracerebroventricular injections of endogenous peptides at hourly intervals which is unsuitable and cumbersome for both livestock and humans. Here, comes the need for synthetic molecules with similar or enhanced functionality but with extended life. Despite kisspeptin being major pharmacological target in reproduction, only few kisspeptin agonist have been disclosed so far.

4.4.1 FTM080

4-fluorobenzoyl-Phe-Gly-Leu-Arg-Trp-NH₂ (Tomita *et al.* 2008) [123]. This molecule showed increased resistance to degradation by matrix metalloproteinases but the effect produced was of lesser duration and even decreased potency than the endogenous KP10 molecule (Matsui and Asami, 2014) [51].

4.4.2 TAK-448 and TAK-683

Incorporation of D-amino acids or replacement of Gly with azaGly residue in the peptide sequence improves their biological potencies by altering their conformational

properties and increases their resistance to metabolic degradation (Mosberg *et al.* 1983; Asami *et al.* 2012) [86, 5]. Substitution of Trp 47 with other amino acids such as serine, threonine, β -(3-pyridyl) alanine or D-tryptophan (D-Trp), produced several azaGly 51 analogs that were resistant to metabolic degradation and among these, the D-Trp 47 analog showed not only high metabolic stability but also excellent KISS1R agonistic activity, although other analogs showed decreased agonistic activities compared with Kp-10 (Asami *et al.* 2012) [5].

Acute effects: Stimulation of GnRH release from hypothalamus.

Chronic effects: Suppression of release of GnRH due to desensitization of GnRH neurons.

Both compounds were active after a single subcutaneous injection and capable of inducing a rapid and long-lasting increase of LH in healthy men. However, no clear dose-response relationship was obtained with either compound (Scott G. *et al.* 2013) [106]. The azaGly6 modification used in the TAK compounds is less efficient in preventing degradation of the Phe5 Gly6 Leu7 sequence. Also, TAK-683 injection unexpectedly led to the regression of ovarian follicles rather than their maturation and ovulation (Goto *et al.* 2014) [45].

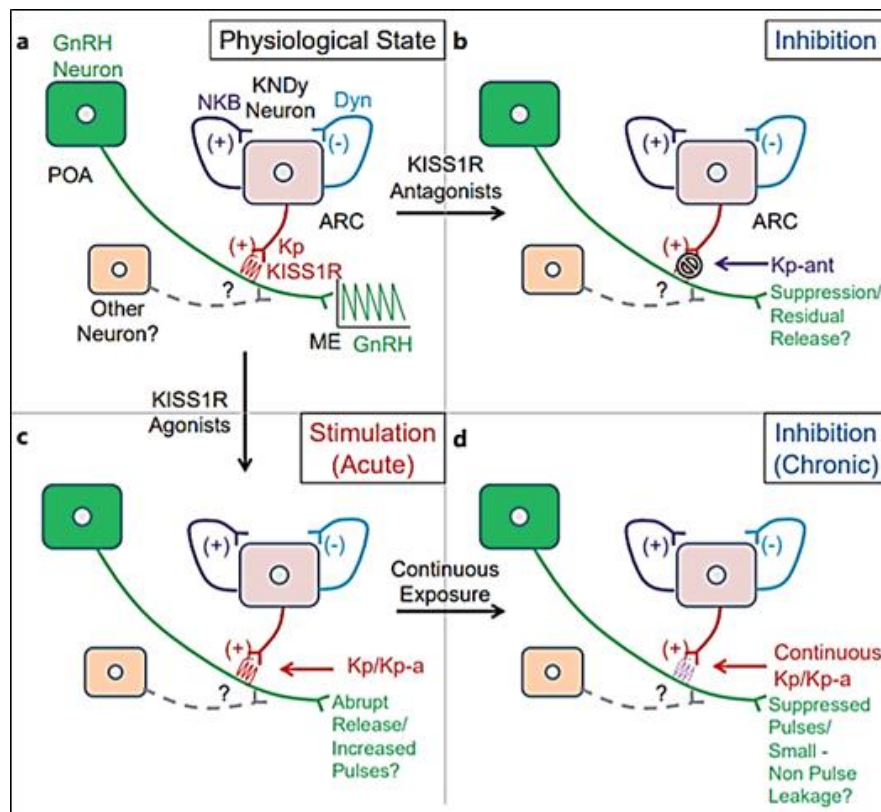


Fig 4: Schematic illustration of hypothalamic GnRH neurons and effects of KISS1R agonists/ antagonists on pulsatile GnRH/LH release. Abbreviations: Dyn= Dynorphin; POA= preoptic area; ME= median eminence; (+)= tonic effect; (-) = inhibitory effect.

4.4.3 Compound-1 to Compound-17

Stepwise targeted modification approach focused on improving pharmacokinetics and pharmacodynamics of the endogenous peptide was used by Beltramo *et al.* 2015 [78]. N-terminal acetylation of Tyr, triazole incorporation at Gly-Leu bond and incorporation of albumin binding motif or covalent linking with larger molecules led to higher proteolytic

stability and slower rates of renal clearance as a result of which half-life and potency of molecule increased to a greater extent. Among all analogues, C-17 came out as outstanding candidate after *in vitro* and *in vivo* studies. Analogs (compounds 2, 3 and 17) designed in this experiment also showed selectivity for KISS1R over NPF1R (>100 times) (receptor for RFRP3).

4.4.4 C2-C7

All the kisspeptin analogs showed enhanced in-vitro and in-vivo activity. Introduction of the albumin-binding motif (*N-palmitoylated-γ-glutamate*) on the N-terminal amine of the triazolepeptide and ω-methylation of Arg⁹ has been shown to dramatically enhance proteolytic stability of Kp10 in blood serum by conferring resistance to trypsin-like proteases and increased the lipophilicity of molecule aiding in crossing blood brain barrier (Decourt *et al.*). Among all, C6 showed best results.

4.5 Kisspeptin antagonists

Kisspeptin agonists were being used in long term to suppress the HPG axis but the initial stimulation of axis by agonists was major problem. Therefore, need arose for development of kisspeptin antagonists. Kisspeptin antagonist analogues were reported by Rosevier *et al.* 2009. Critical amino acid residues were determined that are necessary for receptor binding and antagonistic properties avoiding any residual agonistic effect. Full length of 10 amino acids is necessary for efficient receptor binding and analogues of 5 amino acid length were showing ineffective binding. They noticed that the C-terminal RFamide structure as well as Asn⁴⁶, Trp⁴⁷ and Phe⁵⁰ are critical for receptor binding. In-vitro antagonist assays demonstrated that substitution of Ser⁴⁹ and Leu⁵² with either D-amino acids or Gly⁴⁹ and D-Trp⁵² was the key for antagonistic property and the deletions in the positions of Asn⁴⁶-Trp⁴⁷ led to loss of antagonistic properties. The most effective analogues with these substitutions were peptide 230, 232, 233, 234, 235 and 236. Among all these, peptide 234

came forward as outstanding candidate. Substitution with D-Ala¹ achieved the most complete inhibition.

4.5.1 Peptide 234

- Inhibition of IP stimulation by 10 nM KP10 by 93% with an IC₅₀ of 7nM without showing any intrinsic IP activation.
- Inhibits stimulation of GnRH firing by kisspeptin-10.
- Inhibits pulsatile release of GnRH in pubertal female rhesus macaques.
- Inhibits KP10 LH release in intact male rats and increase in LH levels after castration.
- Inhibits LH pulses in ovariectomized ewes.
- Drawback-requires intracerebroventricular administration for its action.

Sahin *et al.* 2015 [131] conducted experiment using RP-9 and peptide 234 analog in Sprague-Dawley prepubertal female rats as animal model with observations as follows:-

- Central administration of p234 inhibits kisspeptin-induced pubertal advancement and LH increase in the female rats.
- Central administration of p234 inhibits RF9-induced pubertal advancement and increase in LH levels in female rats.
- Central administration of p234 inhibits both kisspeptin and RF9-induced food intake and body weight reductions.

4.6 Therapeutic uses of kisspeptin analogues

Table 4: Summary of effects of Kisspeptin and its analogues on reproductive axis

Molecule	Animal model	Dose and route of administration	Effect on reproductive axis	Reference
Kp-10	Adult non-cyclic ewes	6 nmol, i/v	Increase LH and FSH after each injection	Caraty <i>et al.</i> 2007 [17]
Kp-10	Adult ewes in follicular phase	0.48μmol/h for 8 hours, i/v	Induce LH surge and ovulation	Caraty <i>et al.</i> 2007 [17]
Kp-10	Ewes-Adult non-cyclic	15.2 nmol/h for 24 hours, i/v	Increase LH and E2 Ovulation induction	Sébert <i>et al.</i> 2010 [109]
Kp-10	Ewes-Prepubertal	20 μg/h for 24 hours, i/v	Increase LH pulsatility Induction of ovulation	Redmond <i>et al.</i> 2011 [103]
Kp-10	Cattle- Prepubertal heifers	1mg, i/v	Increase in LH and GH at 27±3 min. and 75±9 min. respectively	Kadokawa <i>et al.</i> 2008 [48]
bKp-53	Beef cattle- Adult cyclic	0.2 and 2 nmol/kg, i/v	0.2 nmol/kg= enhanced follicular growth 2 nmol/kg= follicular growth and ovulation	Naniwa <i>et al.</i> 2013 [88]
Kp-10	Buffaloes-Culture grade oocytes	5, 10, 15 μg/ml in TCM-199	Oocyte maturation (Cumulus cell expansion and 1 st polar body extrusion) 5 μg/ml- 65.32%; 11.70% 10 μg/ml- 73.21%; 22.11% (best) 15 μg/ml- 68.77%; 16.32%	Rajesh <i>et al.</i> 2018 [62]
Kp-10	Swamp buffaloes-mid-luteal phase	1.3 μg/kg body wt. i/v	No increase in LH up to 6 hours	Chaikhun-Marcou <i>et al.</i> 2019 [119]
Kp-10	Mithun cows-Postpartum anestrus	1.3 μg/kg body wt. at 3-day interval for 21 days, i/m	Early resumption of cyclicity (24.64±10.43 days vs 86.56±14.66 days in control group)	Khan <i>et al.</i> 2019 [74]
bKP-10	Murrah buffaloes-Prepubertal and adult cyclic	5, 10 and 15 μg/kg body wt. i/v, i/m	Increased LH (2h) Enhanced follicular growth rate	Pottapenjera <i>et al.</i> 2018 [98]
TAK683	Adult ovariectomized does	500 nmol/kg/ week, s/c	LH pulses abolished	Tanaka <i>et al.</i> 2013 [120]
TAK683	Adult cyclic synchronized does	35 nmol, i/v	Inceased FSH and LH (6 h) Immediate suppression of gonadal steroids Induced ovulation	Goto <i>et al.</i> 2014 [45]
TAK683	Adult cyclic synchronized does	5 μg; i/v, s/c	Increased LH (10 h) Advanced ovulation	Kanai <i>et al.</i> 2017 [59]
FTM080	Adult non-cyclic ewes	0.5, 2.5 and 5 nmol/kg, i/v	Short lasting increase of LH (at all doses)	Whitlock <i>et al.</i> 2015 [129]
C17	Adult non-cyclic ewes	15 nmol, i/v	Increase FSH and LH during 5 and 9 hours	Beltramo <i>et al.</i>

			(approx.) respectively	2015 [78]
C6	Adult ewes in follicular phase	15 nmol, i/m	Increase FSH and LH during 12 hours (approx.) Induce ovulation	Decourt <i>et al.</i> 2016 [15]
C6	Adult non-cyclic ewes	15 nmol, i/m	Increase FSH and LH during 12 h Induce ovulation	Decourt <i>et al.</i> 2016 [15]
C6	Prepubertal gilts (after PG600)	60 nmol, i/m	Increased LH (>16 h) Induced ovulation	Ralph <i>et al.</i> 2018 [99]
C6-	Alpine does (nonbreeding, onset of breeding and breeding season)	15 nmol/doe, i/m	Increases LH and FSH levels Induce fertile ovulation (45% pregnancy rates)	Decourt <i>et al.</i> 2019 [25]
p234	Female rats-prepubertal	Kisspeptin= 50 pmol icv RF9= 10 nmol icv p234= 1 nmol icv	Inhibits Kp and RF9 induced pubertal advancement, increase in LH levels and reduced food intake	Sahin <i>et al.</i> 2015 [131]
p234	Adult female cyclic rhesus monkeys	hKp-10= 1nmol icv p234=10 nmol icv	Inhibits pulsatile GnRH release	Roseweir <i>et al.</i> 2009 [4]
	Adult ovariectomized ewes	p234= 40µg icv	Inhibits LH pulses	
p234, p271, p354 and p356	Anestrus beagle bitches	ckp-10= 0.5 µg/kg body wt. Kp antagonist= 50 µg/kg/h	No antagonistic effect	Albers-Wolthers <i>et al.</i> 2017 [2]

4.7 Other modulators of reproductive axis

Table 5: Summary of molecules showing effect on reproductive axis

Molecule	Mechanism	Effect
RF9	GnIH-R antagonist	Simulates the action of GnRH
Phoenixin	PNX-R	Stimulates release of GnRH
Senktide	NK3R (neurokinin receptor) agonist	Stimulates release of kisspeptin
MLE54901 SB22200	NK3R antagonist	Inhibitory effect on release of kisspeptin
Opioids	Opioid receptors of POA and MBH	Inhibitory effect on GnRH/ LH secretion
Naloxone	Opioid-R antagonist	Antagonizes stress induced inhibition of LH surge

5. Conclusion and Future perspectives

Although many GnRH modulators have been tried to control estrous cycle in farm animals but none of them met the desired levels. Kisspeptin analogues have come forward with encouraging results. Many kisspeptin agonists have been tried by various scientists and among them C6 and C17 compounds showed better results in sheep, goat and laboratory animal model. Although studies have been done on kisspeptin antagonists but only a few molecules are available and p234 have good results in laboratory rodents and ewe model. A standard therapeutic protocol for the use of these novel compounds in farm animals at field level need to be devised.

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