Studies on the Neurotransmitter Regulation of Kisspeptin Neurons

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STUDIES ON THE NEUROTRANSMITTER REGULATION OF KISSPEPTIN NEURONS

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DECLARATION

I hereby declare that the data contained in this thesis are my original work. I have not previously presented any part of this work elsewhere for any other degree.

Shazia Shamas

Dedicated

To

My supervisor and loving Parents,

Whose prayers, love and affections

are source of strength for me in

every step of life

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LIST OF ABBREVIATIONS

˚C	Centigrade
˚N	Degree north
˚E	Degree east
%	Percent
µl/µL	Microliter
µg	Micro gram
µm	Micro meter
mM	Millimol
ALG9	Asparagines-linked glycosylation 9
AM	Ante meridiem
AR	Androgen receptor
ARC	Arcuate nucleus
ARC-KNDy	ARC kisspeptin neurons colocalizing neurokinin and dynorphin
AVPV	Anteroventral periventricular nucleus
BnST	Bed nucleus of stria terminalis
bp	Base pair
BSA	Bovine serum albumin
BW	Body weight
Ca^{+2}	Calcium ions
cDNA	Complementary deoxyribo nucleic acid
CF488	Cyanine-based fluorescent dyes
CNS	Central nervous system
cPVpo	Caudal preoptic periventricular nucleus
Cre GFP	Green fluorescent protein fused to Cre recombinase
Ct	Cycle threshold
CY3	Cyanine dyes
DAB	Diaminobenzidine hydrochloride
DAG	Diacylglycerol
DBB	Diagonal band of Broca
dbl	Double labeled
DEPC	Diethylpyrocarbonate
DMH	Dorsomedial hypothalamus
DNA	Deoxyribo nucleic acid
DPX	Distyrene, a plasticizer, xylene
EAA	Excitatory amino acids
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ERα	Estrogen receptor α
ERαKO	Estrogen receptor α knock out
ERβ	Estrogen receptor β
ERβKO	Estrogen receptor β knock out
ERK	Extra cellular signal regulated kinases
FSH	Follicle stimulating hormone
GABA	Gamma aminobutyric acid

List of Abbreviations

GABAA	Gamma aminobutyric acid A receptor
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GnRH	Gonadotropin-releasing hormone
GPR54	G protein coupled receptor 54
GT-1	Gonadotropin releasing hormone neuronal cell lines
h	Hour
hCG	Human chorionic gonadotropin
HPG	Hypothalamic-pituitary-gonadal
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IGF-1	Insulin-like growth factor 1
IP3	Inositol 1,4,5-trisphosphate
IHH	Idiopathic hypothalamic hypogonadism
ir	Immunoreactivity
im	Intramuscular
ip	Intraperitoneal
iv	Intravenous
KA2	Kainate-2
kg	Kilogram
KP	Kisspeptin
Kp-10	Kisspeptin peptide 10
Kiss-1	K (Hershey 'Kisses'), iss (interim nomenclature for suppressor sequence)1
KISS-1	Kisspeptin protein (human)
Kiss-1	Kisspeptin protein (non-human)
KISS-1R	Kisspeptin receptor (human)
Kiss-1r	Kisspeptin receptor (non-human)
KISS-1	Kisspeptin gene (human)
Kiss-1	Kisspeptin gene (non-human)
KISS-1R	Kisspeptin receptor gene (human)
Kiss-1r	Kisspeptin receptor gene (non-human)
LH	Luteinizing hormone
LD	Long day
L	Length or liter
MC4R	Melanocortin 4 receptor
mg/kg	Milligram (10^{-3} gram)/kilogram
mg/ml	Milligram per milliliter
min	Minute
ml/mL	Milliliter (10^{-3} liter)
mm	Millimeter
NK3R	Neurokinin B receptor
NMDA	N-methyl-D, L-aspartate
NMDAR1	N-methyl-D, L-aspartate receptor subunit 1
NR1	N-methyl-D, L-aspartate receptor subunit 1
OVX	Ovariectomized
p	Short arm of chromosome
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
PeN	Periventricular nucleus
pH	Power of hydrogen
PIP2	Phosphatidylinositol 4,5-bisphosphate
POMC	Pro-opiomelanocortin
POA	Preoptic area
PR	Progesterone receptors
PVN	Paraventricular nucleus
PVpo	Preoptic periventricular nucleus
PLC	Phospholipase C
q	Long arm of chromosome
qPCR	Quantitative polymerase chain reaction
RF-amides	Arginine-phenylalanine-amides
RPM/rpm	Revolutions per minute
RP3V	Rostral periventricular area of third ventricle
RP3V-Kiss1	Kisspeptin neurons located in RP3V
rPVpo	Rostral preoptic periventricular nucleus
RT	Reverse transcriptase
RFRP3	RFamide related peptide 3
SCN	Supra chiasmatic nucleus
SD	Short day
Sec	Second
SEM	Standard error mean
TBS	Tris-buffered saline
TMB	Tetramethylbenzidine
TV	Testicular volume
V	Volume or velocity
VIP	Vasoactive intestinal polypeptide
VMH	Ventromedial hypothalamus
W	Width or watt

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General Abstract

GENERAL ABSTRACT

Background: Kisspeptin is a neuropeptide which acts through the activation of previously orphan G-protein coupled receptor 54 (GPR54). One of the most important functions of kisspeptin neurons is to stimulate gonadotropin-releasing hormone (GnRH) secretion which further regulates reproduction. Although a lot of studies have been done about the kisspeptin regulation of hypothalamic-(brain)-pituitary-gonadal (HPG) axis, data available about the regulation of kisspeptin itself are very scarce. Therefore, the present research work was designed to study the neurotransmitter (glutamate and GABA) regulation of kisspeptin neurons in adult male rhesus macaque under different physiological conditions such as during breeding and non-breeding season and during normal fed and 48 h fasted states. Additionally, expression of gonadal steroid receptor was examined on a specific hypothalamic kisspeptin neuronal population in adult male and female mice to better understand the steroidal regulation of kisspeptin signaling.

Materials and Methods: In monkey studies, hypothalamic blocks containing medio basal hypothalamus (MBH) and preoptic area (POA) were obtained from five intact adult male rhesus monkeys (*Macaca mulatta*), captured from wild during the breeding (n=3; October; TV: 69.00 ± 1.00 ml) and non-breeding season (n=2; July; TV: 12.88 ± 0.31 ml) and from four adult male monkeys during fed (n=2) and 48 h fasting (n=2) states. Hemi-hypothalamic blocks were placed in a fixative for immunocytochemistry and other hemi-hypothalamus was quickly immersed in liquid nitrogen and then placed at -80˚C until RNA isolation. Blood samples were also drawn from animals during breeding and non-breeding seasons to measure plasma testosterone levels and during fed and fasting states to measure plasma testosterone and blood glucose levels. Real time polymerase chain reaction (PCR) was carried out by using specific primers

to quantify the levels of *Kiss1*, *Kiss1r*, *NR1* and glutamic acid decarboxylase 67 (*GAD67*) mRNA during different reproductive seasons and metabolic states. Double label immunofluorescence using specific antibodies against kisspeptin and NR1 was also performed to examine the interaction of kisspeptin neurons with NR1 subunit of N-methyl-D, L-aspartate (NMDA) receptors in hypothalamic MBH of adult male rhesus macaque during the breeding and non-breeding seasons and during normal feeding and 48 h fasting states. In mice studies, ten adult male and ten adult female C57BL/6J mice were used. Dual label chromogen immunocytochemistry was performed on brain sections by using specific primary antibodies directed against kisspeptin and estrogen receptor α (ERα)/ androgen receptor (AR) in RP3V region of male and female mice. Kisspeptin immunoreactivity was revealed by treating the brain sections with diaminobenzidine hydrochloride (DAB) (brown cytoplasmic staining) and ERα/AR immunoreactivity was revealed by treating the brain sections with nickel-enhanced diaminobenzidine hydrochloride (NiDAB) (black nucleus staining).

Results: Our results demonstrated that the expression of *Kiss1*, *Kiss1r* and *NR1* mRNA levels were significantly ($p<0.05$) increased whereas expression of *GAD67* mRNA levels were significantly ($p<0.05$) decreased in the MBH and POA of adult male rhesus monkey during the breeding season and vice versa during the non-breeding season. Plasma testosterone levels ($p<0.01$) and testicular volumes ($p<0.001$) were significantly increased during the breeding season as compared to the non-breeding season. The number of kisspeptin neurons, and the interactions of kisspeptin neurons with NR1 expressing elements were significantly ($p<0.05$) increased during the breeding season as compared to non-breeding season. In the

other experiment, plasma testosterone and blood glucose levels were significantly ($p<0.05$) decreased after short term fasting. Hypothalamic expression of *Kiss1, Kiss1r* and *NR1* mRNA levels were significantly ($p<0.05$) reduced in adult male rhesus monkeys which were fasted for 48 h as compared to those which were fed *ad libitum* while there was no clear difference in *GAD67* mRNA expression levels between the two groups. The number of kisspeptin neurons was significantly ($p<0.05$) reduced after 48 h fasting, and the percentage of kisspeptin neurons expressing NR1 was also reduced after 48 h fasting. In the mice study, kisspeptin neurons residing in rostral periventricular area of third ventricle (RP3V) expressed ERα and AR in both male and female mice. Percentage of RP3V kisspeptin neurons expressing AR was significantly higher ($p<0.05$) in males than females. About 65% and 37% of RP3V kisspeptin neurons expressed ERα in adult male and female mice, respectively. However, this difference in percentage of ERα expressing RP3V kisspeptin neurons between male and female mice was statistically non-significant ($p>0.05$). Percentage of kisspeptin neurons expressing ERα was significantly ($p<0.05$) greater than the percentage of kisspeptin neurons expressing AR in the female mouse. However, percentage of kisspeptin neurons expressing ERα and AR was statistically similar in the male mouse.

Conclusions: Taken together the results of the current research work suggest that kisspeptin signaling is stimulated by glutamate and inhibited by GABA neurotransmitters in adult male rhesus macaques during different photoperiods and metabolic states. This research work strengthens our knowledge about the excitatory (glutamate) and inhibitory (GABA) neurotransmitters regulation of kisspeptin signaling especially in higher primates. In addition, present study extend our

knowledge about the steroid regulation of kisspeptin neurons by demonstrating putative involvement of both androgen and estrogen on a subgroup of hypothalamic kisspeptin population in male and female rodents.

General Introduction

GENERAL INTRODUCTION

Reproduction is a fundamental process that generates continuation of species. Reproduction is governed by the interplay between brain, pituitary and gonads that constitutes the HPG axis. The hypothalamic component of the HPG-axis is furnished by neurons synthesizing and releasing GnRH which is transported to the pituitary gland via hypophysial portal blood circulation. GnRH excites the secretion of two gonadotropins called luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones then drive functions of gonads and the reproduction becomes possible. GnRH is further regulated by several internal and external factors. Regardless of the statement that the major elements responsible for the control of puberty and reproduction had been previously recognized, a major burst through in our knowledge of regulation of the reproductive system took place, when an important regulator of GnRH was discovered (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). This factor is a peptide and is called as "kisspeptin".

Kisspeptin is a neuropeptide encoded by *Kiss1* gene (Lee et al., 1996); it was named after its discovery in Hershey, Pennsylvania, famous for chocolates "Hershey's Kisses". It was initially discovered in human melanoma cell lines as a metastasis suppressor sequence upon transfer of chromosome 6. It was found that these cell lines had reduced metastatic potential of the tumor cells with no effect on cellular proliferation and migration (Lee et al., 1996). Following the discovery of kisspeptin, hybridization technique was used to compare the mRNA expression in metastatic and non-metastatic hybrid clones in which chromosome 6 was transferred. This led to the discovery of a novel *Kiss1*, present only in suppressed melanoma cell lines (Lee et al., 1996). The *Kiss1* is present on the long arm of chromosome 1 (1q32) and contains

four exons (West et al, 1998). However, some factors located on chromosome 6 (Lee et al., 1996; Lee and Welch, 1997; Mitchell et al., 2007) regulate *Kiss1* expression (Murphy, 2005).

The International Committee established for the standardization of nomenclature (http://www.informatics.jax.org/mgihome/nomen/gene.shtml) recommends that human and non-human kisspeptin genes should be denoted as "*KISS1"* and "*Kiss1",* respectively. Whereas, the protein product of these genes should be denoted by the non-italicized version of nomenclature such as "Kiss1" for other species and "KISS1" for humans. "Kisspeptin" is also used to denote the peptide (Gottsch et al., 2009).

The *Kiss1* encodes a larger precursor of 145 amino acids, which undergoes post-translational cleavage and results in the formation of four bioactive forms, kisspeptin-54, -14, -13 and -10 with a different number of amino acids indicated by the suffix (Kotani et al., 2001). The kisspeptin belongs to the RF-amide family of peptides sharing common arginine and phenylalanine residues at the C-terminal (Clements et al., 2001). The shorter peptides of 14, 13 and 10 amino acids are contained by the 54 amino acids peptide and are probably generated by proteolytic degradation within plasma. The comparative significance of the C-terminal and the RF-amide moieties has been established by the effectiveness and affinity of the shorter domains at kisspeptin receptor (Kiss1r) (Kotani et al., 2001). In addition, synthetic kisspeptins missing any one of the 10 residues of the RF-amide chain are incapable to attach and produce a useful receptor response (Kotani et al., 2001). These findings propose that the C-terminal segment is necessary for the binding of receptor and also for its activation.

Kiss1r is a G-protein coupled receptor (aka GPR54) belonging to the rhodopsin family of receptors. It was first described in rats (Lee et al., 1999) and at that time, it was considered an orphan receptor that latterly, was proved a receptor for kisspeptin peptide (Clements et al., 2001; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Subsequent to the discovery of kisspeptin receptor, its gene was found to be present on the chromosome 19p 13.3 (Muir et al., 2001; Ohtaki et al., 2001). The Human Genome Nomenclature Committee suggests that "*KISS1R*" and "*Kiss1r*", respectively, should denote the human and non-human kisspeptin receptor genes. Receptor protein should be denoted by Kiss1r and KISS1R for the non-human and human, respectively (Gottsch et al., 2009). Kisspeptin and Kiss1r are present in various regions of central nervous system (CNS) and peripheral tissues of many vertebrates depending upon the species, gender and physiological status of the individual.

In humans, kisspeptin immunoreactivity has been found in different regions of the CNS. Kisspeptin has been expressed in lamina terminalis, tuberal subdivision of supraoptic nucleus, hypothalamus, stria terminalis and infundibular stalk. In the hypothalamus kisspeptin expression has been observed in POA, dorso medial hypothalamus (DMH), ventro medial hypothalamus (VMH) and the periventricular nucleus (PeN) of human brain (Hrabovszky et al., 2008; 2010). *KISS1R* gene has been found in spinal cord, cerebellum, cerebral cortex, amygdala, basal ganglia, hypothalamus and substantia nigra (Muir et al., 2001).

In the mouse brain, immunocytochemical studies showed that kisspeptin neurons are localized in two distinct hypothalamic regions, one group is present in the arcuate nucleus (ARC) and the second group is in the anteroventral periventricular nucleus

(AVPV). In addition, other less dense and more sprinkled groups of kisspeptin neurons are present in the posterior hypothalamus and DMH. Furthermore, thick kisspeptin neuronal fibers have been identified in PeN, ventral aspect of lateral septum and along ventral retrochiasmatic nucleus. Whereas, sprinkled fibers have been shown in subfornical organ, paraventricular thalamic nucleus, medial amygdala, paraventricular and supraoptic nuclei, amygdala, bed nucleus of the stria terminalis (BnST), locus coeruleus and periaqueductal gray, and absent from suprachiasmatic nucleus (SCN) and VMH (Clarkson and Herbison, 2006; Clarkson et al., 2009a). Sexual dimorphism was observed in the staining of hypothalamic AVPV kisspeptin neurons of male and female mice. Number of kisspeptin cell bodies in AVPV region is 10 times higher in females as compared to males (Clarkson and Herbison, 2006). The *Kiss1r* mRNA is found to be present in the CNS (Funes et al., 2003) and predominantly on GnRH neurons (Han et al., 2005; Messager et al., 2005). *Kiss1r* is also expressed in the dentate gyrus of the hippocampus, the periventricular region of the posterior hypothalamus and is absent from ARC nucleus (Herbison et al., 2010).

In rats, *Kiss1* is present throughout the CNS including pons, hypothalamus, medulla, midbrain, cerebral cortex and spinal cord (Irwig et al., 2004; Brailoiu et al., 2005; Kauffman et al., 2007a) and in the pituitary (Richard et al., 2008). By the use of immunocytochemistry kisspeptin neurons have been recognized in the hypothalamic ARC, VMN and paraventricular nucleus (Brailoiu et al., 2005). *Kiss1r* was initially reported in midbrain, pons, hippocampus, cortex, amygdala, striatum, frontal cortex, hypothalamus and in some peripheral organs (liver and intestine) (Lee et al., 1999). The *Kiss1r* expression has also been noticed in diagonal band of Broca (DBB), medial

septum, medial preoptic area, lateral hypothalamus and anterior hypothalamus (Irwig et al., 2004).

In the sheep brain, kisspeptin immunoreactivity has been predominantly recognized in ARC of the hypothalamus. Additionally, kisspeptin neurons are found in the VMH, PeN, DMH, caudal region of the paraventricular nucleus and medial POA (Franceschini et al., 2006; Pompolo et al., 2006). Kisspeptin nerve fiber density is the highest in the median eminence (ME), POA, ARC, VMH, PeN and DMH (Franceschini et al., 2006; Pompolo et al., 2006). *Kiss1r* mRNA has been observed to be found in pituitary gonadotropes (Smith et al., 2008).

In the rhesus macaque brain, kisspeptin cell bodies have been found in ARC but absent in POA (Shibata et al., 2007; Ramaswamy et al., 2008). Kisspeptin neuronal projections are identified all over the MBH and few are found in the POA (Ramaswamy et al., 2008). Kiss1r has also been found in these hypothalamic nuclei (Shahab et al., 2005; Shibata et al., 2007).

Kisspeptin neurons are also found in many other species such as goat, horse, pig and hamster (Estrada et al., 2006; Decourt et al., 2008; Ohkura et al., 2009; Tomikawa et al., 2010). In goat brain, kisspeptin neurons are found in the ARC whereas dense neuronal fibers are present in ME (Ohkura et al., 2009). In pigs, *Kiss1r* expression has been observed in ARC and PeN (Li et al., 2008; Tomikawa et al., 2010). In Syrian hamsters, *Kiss1* expression has been recognized only in the ARC (Revel et al., 2006) while, in Siberian hamster kisspeptin expression has been noticed in both AVPV and ARC (Mason et al., 2007; Greives et al., 2007). In horse brain, kisspeptin neurons are present in ARC, DMH and preoptic periventricular zone of the hypothalamus (Decourt et al., 2008).

Kisspeptin neurons are also present in non-mammalian brain. Expression of *Kiss1* mRNA has been observed in the hindbrain and midbrain of frog (Lee et al., 2009). *Kiss1r* is also present in the hypothalamus, forebrain and the hind brain (Lee et al., 2009). In the piscine species such as medaka, gold fish, zebra fish and sea bass, *Kiss1* mRNA is identified in the hypothalamic periventricular region and thalamus (Kanda et al*.,* 2008; Li et al., 2009; Mechaly et al., 2009). In tilapia, cobia and fathead minnow, *Kiss1r* expression has been observed in the hypothalamus, midbrain and POA (Parhar et al*.,* 2004; Filby et al., 2008).

Kisspeptin acts through the activation of GPR54 or Kiss1r as shown in Figure i. Upon binding of kisspeptin, Kiss1r activates phospholipase C (PLC), which converts phosphatidylinositol bisphosphate (PIP2) into inositol-1, 4, 5-triphosphate (IP3). In turn, IP3 releases intracellular calcium ions (Ca^{+2}) from the endoplasmic reticulum, which causes changes in ion channel permeability and depolarization of cells. Furthermore, increase in PIP2 hydrolysis causes diacylglycerol (DAG) formation and phosphorylation of kinases (Kotani et al., 2001; Castellano et al., 2006).

After the discovery of kisspeptin, it has been found to be associated with several tasks. One of the most fundamental roles played by kisspeptin is the regulation of HPG axis and hence the reproduction. A rise in plasma LH, FSH and testosterone levels evidenced kisspeptin stimulation of HPG axis after the peripheral metastin administration in adult men (Dhillo et al., 2005). This increase in gonadotropin secretion has also been observed in laboratory animals (Gottsch et al., 2004; Navarro et al., 2004; Plant et al., 2006). Moreover, central kisspeptin-54 infusion in POA, ARC and PVN in adult male rats causes rise in plasma testosterone and LH levels (Patterson et al., 2006). Central or peripheral kisspeptin-10 (Kp-10; minimal active

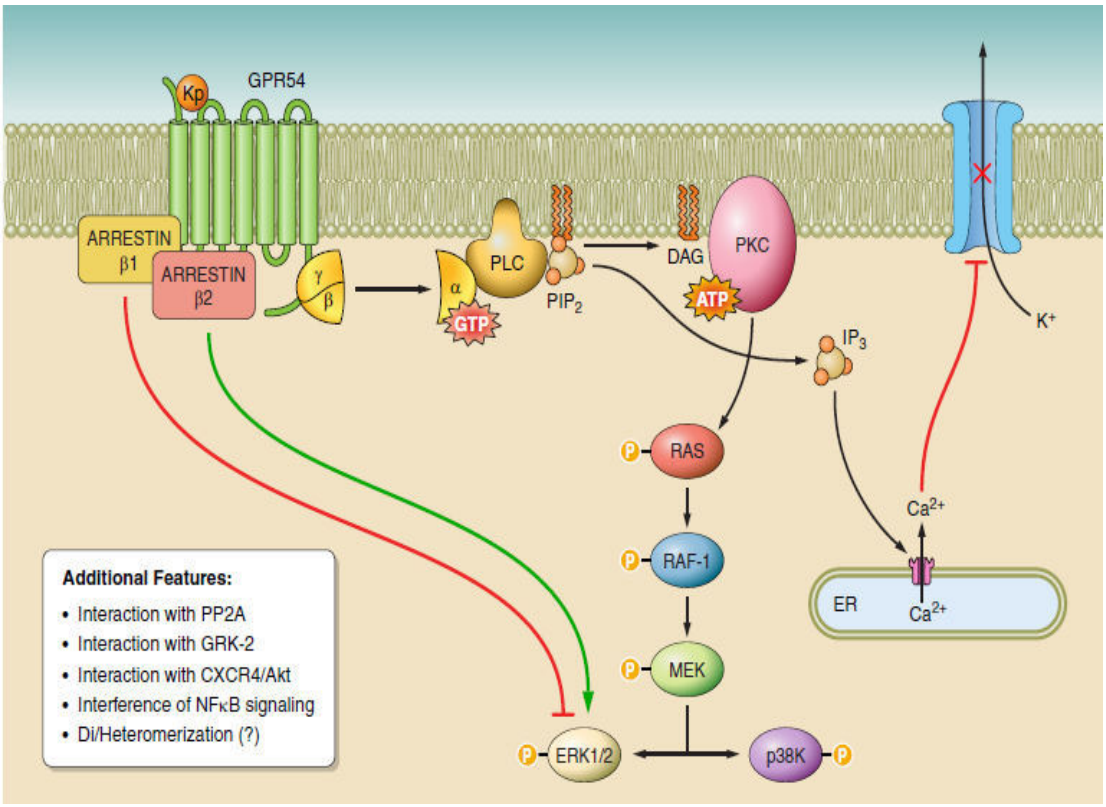

Figure i. A diagram showing intracellular signaling pathways which are activated upon binding of kisspeptin with its receptor. Upon binding of kisspeptin, GPR54 activates PLC, which converts PIP2 into IP3. In turn, IP3 releases intracellular calcium ions which cause changes in ion channel permeability and depolarization of cells. Furthermore, increase in PIP2 hydrolysis causes DAG formation and phosphorylation of kinases. In addition, GPR54 also regulates ERK phosphorylation through activation of arrestin β1 and arrestin β2 (source: Pinilla et al., 2012).

fragment of kisspeptins) treatment causes hypothalamic pulsatile GnRH release, as revealed by LH release, whereas acyline (GnRH antagonist) treatment blocks Kp-10 induced secretion of LH in agonadal rhesus monkeys (Plant et al., 2006). Moreover, centrally administered kisspeptin resulted in the rise of peripheral FSH and LH levels in pre-pubertal and adult female and male mice and rats (Gottsch et al., 2004; Navarro et al., 2004; Thompson et al., 2004; Navarro et al. 2005a; 2005b). In many other species such as goats, cows, sheep, pigs and horses kisspeptin also stimulates the LH secretion (Abbara et al., 2013). These findings reveal that central or peripheral administration of metastin and Kp-10 causes the release of LH, FSH and testosterone in humans and laboratory animals.

Kisspeptin regulation of the reproduction was first noticed in idiopathic hypogonadotropic hypogonadism (IHH) patients showing loss of function mutations of kisspeptin receptor gene (Seminara et al., 2003; de Roux et al., 2003) and later it was also confirmed in transgenic mouse (Funes et al., 2003; Kauffman et al., 2007a; Lapatto et al., 2007). Since then kisspeptin has been found to be critical for the initiation of puberty and maintenance of reproductive functions in adults. Kisspeptin regulates the secretion of GnRH (Dhillo et al., 2005, 2007) in a large number of species such as mice (Gottsch et al., 2004; Messager et al., 2005), rats (Navarro et al., 2004), sheep (Messager et al., 2005; Caraty et al., 2007) and monkeys (Plant et al., 2006). Kisspeptin acts on the GnRH neurons, as revealed in sheep (Messager et al., 2005), goats (Hashizume et al., 2010), monkeys (Keen et al., 2008) and pigs (Lents et al., 2008). Further studies have revealed that there is co-expression of GnRH neurons and kisspeptin receptors (Irwig et al., 2004; Han et al., 2005). By using transgenic mouse models like *Kiss1r* and *Kiss1* knockout, it has been found that functional

kisspeptin receptors are necessary for GnRH release and for LH and FSH release (Seminara et al., 2003; Lapatto et al., 2007). In order to examine the interaction of kisspeptin and GnRH neurons, many experiments have been carried out which showed that kisspeptin increased the GnRH neuron firing rate (Dumalska et al., 2008; Liu et al., 2008) and GnRH neuronal activity. This notion is supported by the observation of c-fos expression (Irwig et al., 2004) and GnRH neurons depolarization by kisspeptin administration (Han et al., 2005; Zhang et al., 2008). Close interaction between kisspeptin and GnRH has been shown in primates. About 25-50% contacts have been identified between GnRH cell bodies and kisspeptin axons in the MBH. On the other hand, kisspeptin and GnRH axons are connected in the ME, while axo-axonal contacts are infrequent in this region (Ramaswamy et al., 2008). It has been suggested that kisspeptin may regulate GnRH secretion non-synaptically in ME. Additional proof of this observation is evidenced from kisspeptin release in the ME in a pulsatile manner and its synchronization with the pulsatile discharge of GnRH in this region (Keen et al., 2008).

Among all the neuropeptides and neurotransmitters, which regulate secretion of GnRH, kisspeptin is the most powerful stimulator of GnRH secretion. This observation is supported by the electrophysiological studies that GnRH neurons respond to Kp-10 (Han et al., 2005) and central injection of Kp-52 induces c-fos expression in GnRH neurons (Irwig et al., 2004). In addition, kisspeptin receptors are also present on GnRH neurons (Irwig et al., 2004; Navarro et al., 2005a). In many species, peripheral or central kisspeptin administration induces GnRH/LH release (Gottsch et al., 2004; Dhillo et al., 2005; Messager et al., 2005; Shahab et al., 2005). *Kiss1r* knockout mice (Funes et al, 2003; Seminara et al., 2003) are unable to achieve

puberty due to defective GnRH release. Although there is significant evidence that kisspeptin neurons are imperative regulators of GnRH secretion, there are limited data available about their own regulation and morphological characteristics. After the identification, that kisspeptin is a powerful regulator of GnRH secretion, it has become obligatory to examine anticipated machinery and neuronal pathways that regulate kisspeptin signaling pathway. Hard work in this front permits explaining the chain of commands and ultimate interactions of kisspeptins with other neurotransmitters. Unfortunately, this particular area of kisspeptin regulation gained little attention during the past. Current studies propose that kisspeptin neurons are regulated by different internal and external environmental cues such as photoperiod, energy status and sex steroids of the individual (reviewed by Kitahashi and Parhar, 2013) as shown in Figure ii.

A yearly cycle of reproduction, where a discrete period of sexual activity is followed by a period of inactivity, is a commonly occurring phenomenon in mammals. It results in the births of offspring during most favourable environmental settings. Therefore, environmental factors such as rainfall, day length, temperature and food supply take part in determining the beginning of breeding season. In seasonal breeding animals, the yearly cycle of day length is the most important cause of synchronization of reproductive activities during the breeding season (Turek and Campbell, 1979).

Several species of Old and New World monkeys also exhibit annual cycle of reproductive functions in their natural environment (Lancaster and Lee, 1965). Many authors have described that several external factors play an imperative role in determining the beginning of breeding season in free ranging colonies in their

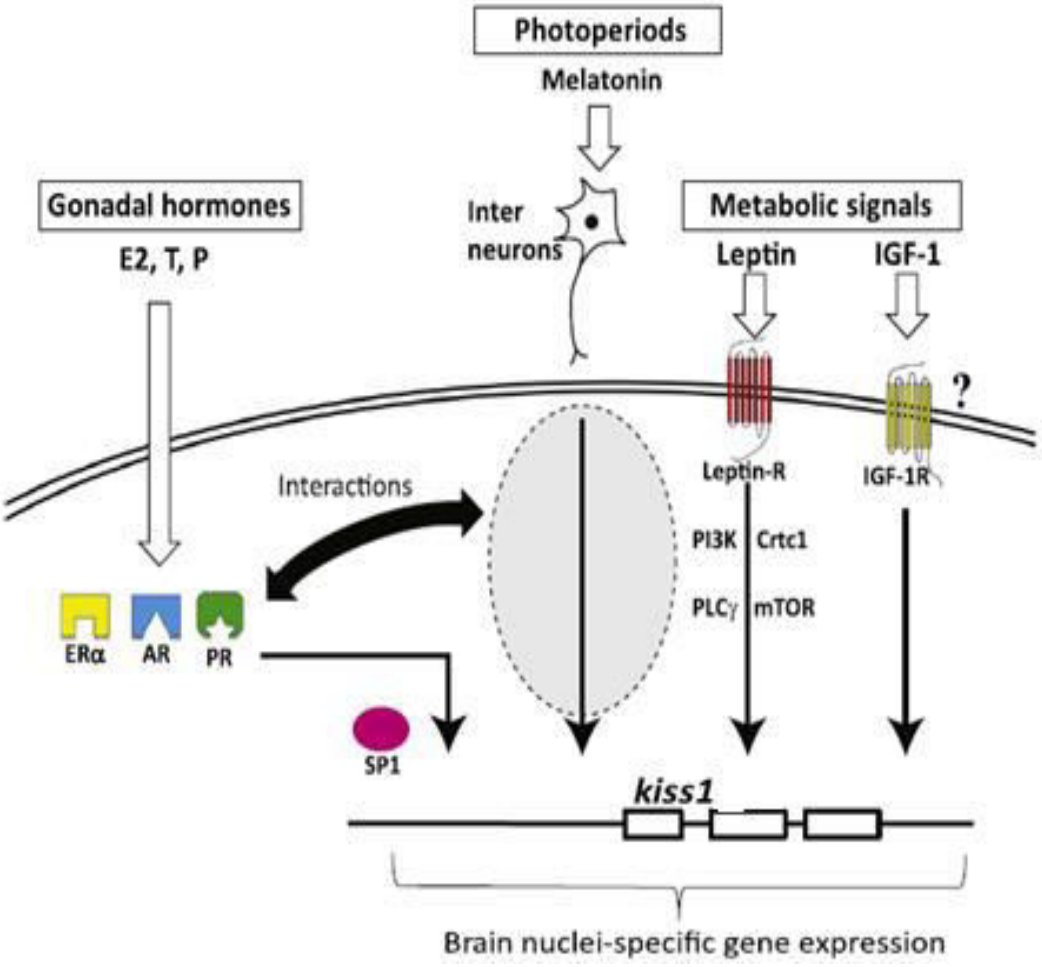

Figure ii. A diagram showing regulatory mechanisms of kisspeptin gene. Metabolic signals, gonadal steroids and photoperiods regulate expression of kisspeptin gene (source: Kitahashi and Parhar, 2013).

natural habitat and semi natural breeding colonies (Vandenbergh and Vessey, 1968; Drickamer 1974; Varley and Vessey, 1977). Koford (1965) described that seasonal breeding is principally due to the annual changes in environmental factors and is not regulated by internal regulatory mechanisms. However, the existence of seasonal reproduction in laboratory kept monkeys (Keverne and Michael, 1970; Wickings and Nieschlag, 1980) indicates that the seasonal breeding is also controlled by internal regulatory mechanisms. Monkeys are reproductively active during September-January. Mating and conceptions occur during this time, followed by the births of offspring. During summer, many female monkeys become amenorrheic (Keverne and Michael, 1970). There is an annual cycle of sexual activity and testicular volume in free-ranging male rhesus macaque (Sade, 1964; Zamboni et al., 1974).

It has been suggested that photoperiodic signal is conveyed to the reproductive axis through kisspeptin neurons. At night, pineal gland secrets a hormone melatonin which mediates photoperiodic signal. In hamsters, AVPV and ARC kisspeptin neurons are regulated by photoperiods (Simonneaux et al., 2009). There are more number of *Kiss1* mRNA expressing neurons under long day (LD) conditions as compared to short days (SD) in Syrian hamsters (Revel et al., 2006; Ansel et al., 2010). However, pinealectomy results in the rise of ARC *Kiss1* mRNA levels but not the AVPV *Kiss1* mRNA (Ansel et al., 2010). In Siberian hamster, *Kiss1* gene regulation by photoperiod is different than Syrian hamsters. In Siberian hamsters, under SD conditions the expression of *Kiss1* is lowered in the ARC, however in the AVPV *Kiss1* gene expression is amplified (Mason et al., 2007; Simonneaux et al., 2009). It has been proposed that in Syrian hamsters, the seasonal melatonin signals are intervened by the DMH (Maywood et al., 1995), while in the Siberian hamsters, the

SCN conveyed the seasonal melatonin signals (Bartke et al., 1992). As a result, the variation in the regulation of *Kiss1* gene in these two species imitates the difference in the seasonal melatonin integrative mechanisms (Simonneaux et al., 2009).

On the other hand, in OVX ewes, which are SD breeders *Kiss1* mRNA expression increases under SD conditions as compared to LD conditions. This finding suggests that photoperiod regulates *Kiss1* gene in the ewe to initiate the breeding season (Smith et al., 2007). However, kisspeptin neurons do not express melatonin receptors in ewe, which shows a possible indirect effect of melatonin on kisspeptin neurons (Li et al., 2011).

It has been emerging that the metabolic status information is conveyed to GnRH neurons through kisspeptin (Roa et al., 2008; George et al., 2011; Wahab et al., 2011). A significant amount of energy is necessary for the maintenance of reproductive functions specifically ovulation in females. Negative energy balance results in the suppression of ovulation (Brown et al., 2008; Donato et al., 2011). The metabolic state regulates kisspeptin gene expression. Short term fasting decreases the expression of *Kiss1* and *Kiss1r* in adult mice (Luque et al., 2007) and monkey (Wahab et al., 2010). It has been well established that food deprivation reduces the *Kiss1* mRNA expression in both adult and pre-pubertal male and female rats (Roa et al., 2008, 2010). The kisspeptin receptor expression also decreases in lean rats as compared to normal weight rats (Castellano et al., 2005). Kisspeptin administration, after short term fasting, restores reproductive function (Navarro et al., 2005b; Castellano et al., 2009). Metabolic regulation of kisspeptin neurons is likely mediated through other hormones such as leptin, ghrelin etc. Leptin stimulates kisspeptin expression in ARC. Leptin gene knockout (ob/ob) male mice demonstrate reduced *Kiss1* expression in

ARC, and this reduced expression of kisspeptin is re-established by leptin treatment (Smith et al., 2006). About 42% of kisspeptin neurons in ARC co-localize leptin receptors (Smith et al., 2006), whereas AVPV kisspeptin neurons lack leptin receptors (Cravo et al., 2011; Smith et al., 2006). In guinea pig, electrophysiological studies have demonstrated the direct stimulation of ARC kisspeptin neurons by leptin (Qiu et al., 2011). In pre-pubertal female rats, *Kiss1* gene has also been shown to be regulated by insulin like growth factor-1 (IGF-1) (Hiney et al., 2009). It stimulates *Kiss1* gene expression in the AVPV but not in the ARC (Hiney et al., 2009). Whether it acts directly or indirectly on kisspeptin neurons is not clear. Although the expression of *Kiss1* gene in AVPV and ARC regions of female rats is stimulated by ghrelin, the presence of ghrelin receptors on kisspeptin neurons remains to be elucidated (Forbes et al., 2009). Kisspeptin neurons present in the ARC and AVPV region co-localize melanocortin 4 receptor (MC4R), signifying some function of pro-opiomelanocortin (POMC) in *Kiss1* gene regulation (Cravo et al., 2011).

Vasopressin and vasoactive intestinal peptide (VIP), two classical transmitters produced and released from SCN, have no effect on RP3V-Kiss1 neurons (located in the RP3V) (Ducret et al., 2010). Another important neuropeptide, RFRP3, concerned with reproduction, has no effect on RP3V-Kiss1 neurons (Ducret et al., 2010). ARC-KNDy neurons (located in the arcuate nucleus co-localizing kisspeptin, dynorphin and neurokinin B) are strongly modulated by neuropeptide, neurokinin B (Navarro et al., 2011). Neurokinin B (NKB) and neurokinin B receptor (NK3R) agonists are potent activators of KNDy neurons, providing a mechanistic interpretation of how NKB sustains normal reproductive functions. In vivo, in the ovariectomized goat, intracerebroventricular infusions of NKB induce multiunit volleys in the vicinity of

ARC-KNDy cells whereas the dynorphin, which is also co-localized in KNDy neurons, inhibits multiunit activity. These findings suggest that the effect of NKB occurred through NKB-induced depolarization of ARC-KNDy cells (Wakabayashi et al., 2010). However, the effect of NKB and dynorphin on *Kiss1* expression needs to be elucidated further.

Estradiol and testosterone have been shown to regulate kisspeptin gene (Smith et al., 2005a; Smith et al., 2005b). Sex steroid hormones induce both up and down-regulation of kisspeptin gene which result in the positive and negative feedback control of the HPG axis. It had been a mystery for a long time that how steroids regulate HPG axis, a major burst through occurred in the ground of reproductive neuroendocrinology when it was identified that steroids regulate kisspeptin gene (Oakley et al., 2009). It has been found that *Kiss1* gene regulation by estradiol is nuclei dependent in rodents. In the ARC region, expression of *Kiss1* gene is inhibited by estradiol, in both female and male mice, but is increased in the AVPV with estradiol treatment (Smith et al., 2005a; Smith et al., 2005b). Similarly, up and down regulation of the *Kiss1* mRNA by gonadal steroids has been shown in AVPV and ARC nuclei of Syrian hamster (Ansel et al., 2010). Consequently, AVPV and ARC populations of kisspeptin neurons mediate positive and negative feedback effects of steroid hormones in both mice and hamsters, respectively.

These negative and positive feedback effects of steroid hormones in ARC and AVPV, respectively, are mediated by ERα (Smith et al., 2005a). Nearly all of the kisspeptin neurons in AVPV and ARC express ERα in female mice (Cravo et al., 2011) and rats (Smith et al., 2006). A distinct proportion of ARC and AVPV kisspeptin neurons also express estrogen receptor β (ERβ) in female mice (Smith et al., 2005a) and rats

(Smith et al., 2006). However, ERβ knock out (ERβ-KO) mice has been found to show normal response to estradiol treatment. Therefore, it has been suggested that ERβ might not be involved in the *Kiss1* gene regulation while estrogen receptor α knock out (ERα-KO) mice have reduced negative and positive estradiol effects on the level of *Kiss1* mRNA (Smith et al., 2005a).

The expression of *Kiss1* gene is also regulated by androgens in male mice. Androgens working through AR negatively regulate the *Kiss1* gene in the ARC region. Particularly, AVPV is sexually dimorphic in mice, having large number of kisspeptin neurons in females than males. Consequently, kisspeptin neurons in the AVPV may play limited role in males. Sex differences in the kisspeptin neuronal number begin on postnatal day 12 (Semaan et al., 2010), probably due to a rise in peripheral steroid hormone levels or steroid receptor expression levels.

In ewe, kisspeptin neurons only in ARC region are sensitive to steroid hormones. Ovariectomy (OVX) causes an increase in ARC *Kiss1*gene expression which proceeds to normal after estradiol administration (Smith et al., 2007), demonstrating kisspeptin neurons in the ARC transduce negative feedback effects of estradiol in the ewe. It has been suggested that this negative feedback effect is mediated by ERα, as nearly all of the kisspeptin neurons in the ARC co-localize ERα (Franceschini et al., 2006). In addition, the progesterone also inhibits the expression of *Kiss1* in the ewe. In OVX ewe, kisspeptin gene expression increases and this increase in expression is restored by progesterone administration. Almost eighty six percent of kisspeptin neurons in the ARC area express progesterone receptors (PR) (Smith et al., 2007). In ewe, kisspeptin neurons in the ARC region are also the center of GnRH/LH surge (Estrada et al.,

2006). It seems that distinct ARC kisspeptin neuronal sub-populations mediate positive and negative feedback effects of estradiol in the ewe (Smith et al., 2008).

Kisspeptin neurons are subjected to an afferent neurotransmitter regulation also. The proposed neurotransmitters which may regulate kisspeptin neurons are excitatory (glutamate) and inhibitory (GABA). It is evidenced that kisspeptin interacts with GABA and glutamate (d'Anglemont de Tassigny et al., 2010; Kurian et al., 2012). Excitatory amino acids (EAA) stimulate the reproductive axis of several mammals. EAA acts through two types of receptors, i.e. metabotropic and ionotropic. Ionotropic receptors are ligand-gated ion channels while the metabotropic receptors are G-protein-coupled receptors (Hollmann and Heinemann, 1994; Petralia and Wenthold, 1995). The ionotropic receptors are involved in neuroendocrine processes (Petralia and Wenthold, 1995). The ionotropic receptors are further divided into three classes each is named according to its ligand: NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate. Glutamate neurotransmitter is an important regulator of GnRH neuronal activity (Olney et al., 1976; Ondo et al., 1976). Intravenous administration of N-methyl-D, L-aspartate can result in precocious puberty in immature rats (Urbanski et al., 1987) and activation of ionotropic glutamate receptors plays an important role in both surge (Ping et al., 1994) and pulse (Bourguignon et al., 1989) modes of GnRH secretion. Administration of glutamate can enhance circulating LH levels and this effect is exerted centrally, since exposure of pituitary slices to glutamate or glutamate injection directly into the hypophysis do not alter LH release (Jennes et al., 2002). Certainly, glutamate can regulate GnRH neuronal activity at the level of dendrites and cell bodies of GnRH neurons and exhibit immunoreactivity for ionotropic glutamate receptors (Gore et al., 1996; Eyigor

and Jennes, 1996). Another important site of action for glutamate is the ME. Glutamatergic axons are apposed to GnRH nerve terminals in ME (Kawakami et al., 1998; Lin et al., 2003) and express immunoreactivity for the kainate-2 (KA2) and NMDA receptor subunit 1 (NMDAR1) (Kawakami et al., 1998). In addition, agonists of ionotropic glutamate receptors and glutamate can induce Ca^{2+} dependent GnRH release from superfused ME fragments (Kawakami et al., 1998). In mammals, NMDA receptor is the most important type of glutamate receptors that is necessary for the secretion of GnRH (Jennes et al., 2002; Claypool et al., 2000; Plant et al., 1989). Gonadotropin releasing hormone neuronal cell lines (GT-1) express NMDA receptors (Mahachoklertwattana et al., 1994). Although the *NR1* mRNA has been expressed by GnRH neurons (Ottem et al., 2002), lack of c-fos immunoreactivity in GnRH neurons subsequent to NMDA administration (Saitoh et al., 1991; Lee et al., 1993) indicates that GnRH neurons may also be excited by NMDA through intermediate neuronal pathways. Recently it has been shown that in the ARC of mice, NMDA receptor agonist administration, causes an increase of c-fos expression in kisspeptin cells, thus suggesting the stimulation of kisspeptin neurons by glutamate (d'Anglemont de Tassigny et al., 2010).

Initial attempts to explain the chain of commands and potential interactions between kisspeptinergic and glutamatergic neurotransmission involved the testing of the effects of NMDA (40μm) on ARC kisspeptin neurons in mice. It was found that NMDA induces firing activity in both loose patch and whole cell patch recordings, as shown in *Kiss1*-CreGFP knock-in mice (Gottsch et al., 2011). These findings suggest that kisspeptin act downstream of glutamate afferents. It has been found recently that NMDA acts on GnRH neurons both directly and indirectly. NMDA acts on GnRH

nerve terminals through kisspeptin signaling pathway in male mouse while acts on GnRH cell bodies independent of kisspetin signaling pathways. In current times, it has been found that peripheral administration of NMDA needs kisspeptin signaling pathway to arouse release of LH in both pre-pubertal and adult male mice, and increases c-fos expression in kisspeptin neurons in adult male mice (d'Anglemont de Tassigny et al., 2010).

GABA is a key inhibitory neurotransmitter in the hypothalamus as well as CNS (Decavel and Van Den Pol, 1990). Leranth et al. (1985) demonstrated glutamic acid decarboxylase (GAD) positive axons forming synaptic contacts with GnRH positive neurons. GnRH neurons express GABAA receptors (Petersen et al., 1993) and results of in vivo micro infusion experiments show that GABA inputs on GnRH cell bodies put forth an important inhibitory influence on the secretory activity of GnRH neurons in numerous species (Herbison et al., 1991). Nevertheless, the role of GABAA receptor signaling on the electrophysiological properties of mature GnRH neurons is somewhat controversial in literature. Defazio et al. (2002) have revealed that endogenous synaptic stimulation of GABAA receptors can be excitatory to mature GnRH neurons due to their unusual intracellular ionic milieu. In contrast, other investigators found evidence indicating that GABA exerts inhibitory effects on adult GnRH neurons (Han et al., 2002). This argument has been supported by the evidence that GABA depolarizes GnRH neurons only when all of the glutamatergic receptor signals are blocked in the brain slice preparation (Moenter and DeFazio, 2005). These findings show the significance of the equilibrium between glutamate and GABA neurotransmission in the firing activity of GnRH neurons.

There is evidence that kisspeptin neurons interact with GABA (Kurian et al., 2012) and glutamate (d'Anglemont de Tassigny et al., 2010). This point of view is supported by the observation that in the ARC of mice, NMDA receptor agonist administration, causes an increase of c-fos expression in kisspeptin cells, suggesting the stimulation of kisspeptin by NMDA (d'Anglemont de Tassigny et al., 2010). Similarly, an interaction of kisspeptin with GABA has also been shown. This finding is supported by the observation that in prepubertal monkeys, bicuculline, GABA antagonist administration, arouses kisspeptin release (Kurian et al., 2012).

That the kisspeptin signaling is strongly modulated by excitatory and inhibitory neurotransmitters, is further supported by the observation of the activation of ARC-KNDy neurons (Cheng et al., 2010) by NMDA and inhibition of KNDy neurons by GABA (Kurian et al., 2012; Gottsch et al., 2011; Qiu et al., 2011). Similar to the ARC-KNDy cells, RP3V-Kiss1 (located in the rostral periventricular area of third ventricle) neurons are also strongly activated by glutamate and inhibited by GABA (Ducret et al., 2010).

Since the discovery of kisspeptin, its function in the regulation of reproductive axis has been well established in various species. It plays an imperative role in initiation of puberty and maintenance of reproductive function in adults. Many other functions have also been assigned to kisspeptin neurons. Still relatively little interest has been paid to the regulation of kisspeptin signaling itself. It is well established that kisspeptin expression is altered in various conditions where HPG axis is affected (Oakley et al., 2009). For example, an alteration of kisspeptin expression has been reported in response to steroid hormones such as estradiol and testosterone (Oakley et al., 2009), photoperiodic cues (Chalivoix et al., 2010), metabolic signals (Fernandez-

Fernandez et al., 2006; Wahab et al., 2010) and peptides (Backholer et al., 2010a, b). However, the mechanisms that link metabolic status and photoperiods with kisspeptin are unknown.

Collectively, recent evidences propose that kisspeptin is the imperative regulator of GnRH secretion. Electrophysiological studies have revealed that GnRH neurons respond to Kp-10 (Han et al., 2005). A central injection of Kp-52 induces expression of c-fos in GnRH neurons (Irwig et al., 2004). In addition, GnRH neurons also express kisspeptin receptors (Irwig et al., 2004; Navarro et al., 2005). In many species, peripheral or central kisspeptin administration induces the GnRH/LH release (Gottsch et al., 2004; Dhillo et al., 2005; Messager et al., 2005; Shahab et al., 2005). Mice having targeted deletions of kisspeptin receptor gene (Funes et al, 2003; Seminara et al., 2003) are unable to achieve puberty due to defective GnRH release. Although many studies have been done about the kisspeptin regulation of HPG axis, data available about the regulation of kisspeptin itself are very scarce.

We studied the role of seasonal variation and metabolic information in influencing glutamatergic-kisspeptin and GABA-kisspeptin interactions and subsequent regulation of kisspeptin signaling in the adult male rhesus macaque, a representative higher primate. However, the influence of different gonadal steroids on kisspeptin signaling was examined in a mouse model.

Objectives

The present research work was designed to study the regulation of kisspeptin neurons by two afferent excitatory (glutamate) and inhibitory (GABA) neurotransmitters in adult male rhesus macaques under different physiological conditions such as during the breeding and non-breeding seasons and during normal fed and 48 h fasted states.

Additionally, expression of gonadal steroid receptor was examined on a specific hypothalamic kisspeptin neuronal population in adult male and female mice to better understand the steroidal regulation of kisspeptin signaling. Presently, there are no data available about the regulation of kisspeptin signaling under different physiological conditions in primates. Until now, to the best of our knowledge, there are no studies available to show whether kisspeptin neurons express NMDA receptors and *GAD67* expression in the hypothalamus of primates in different physiological conditions. Similarly, morphological interaction between NR1 expressing elements and kisspeptin neurons has not been studied. This interaction has been assessed by examining NR1 (an NMDAR marker) positive cell appositions on kisspeptin neurons. Interactions with GABA have been assessed by examining correlative changes in the expression of *GAD67* (catalyzing GABA synthesis) and *Kiss1* gene in the hypothalamus by quantitative real time PCR. These objectives were achieved by doing following experiments

1. By the assessment of direct innervations of kisspeptin neurons by NMDA afferent pathways by using immunocytochemistry in adult male rhesus macaque brain during the breeding and non-breeding seasons, and fed and fasting conditions.
2. By quantifying and comparing the hypothalamic kisspeptin (*Kiss1*), kisspeptin receptor (*Kiss1r*), *NR1* and *GAD67* mRNA contents by using real time PCR in adult male rhesus macaque brain during the breeding and non-breeding seasons, and fed and fasting conditions.
3. By studying the steroid receptor expression in the RP3V kisspeptin neurons of male and female mice by doing dual label chromogen immunocytochemistry.

General Materials and Methods

GENERAL MATERIALS AND METHODS

Animals

In total of nine adult intact male rhesus monkeys (*Macaca mulatta*; 7.2-13 kg body weight), ten adult male and ten adult female C57BL/6J mice were used. Monkeys were used to study the regulation of kisspeptin signaling by two afferent excitatory (glutamate) and inhibitory (GABA) neurotransmitters by the quantification and comparison of *Kiss1, Kiss1r, NR1* and *GAD67* mRNA in the POA and MBH, and the interaction of kisspeptin neurons with NR1 subunit of NMDA receptor during different reproductive seasons and metabolic states. Mice were used to study the co-localization of kisspeptin neurons with ERα and AR in RP3V region.

Monkeys. Five wild and four laboratory kept adult male rhesus monkeys were used in the current research work. Among five wild animals, three were captured from Ayubia National Park, KPK, Pakistan; 33.86˚N, 73.13˚E during the breeding season i.e. October. Whereas, two wild animals were captured from Margalla Hills National Park, Islamabad, Pakistan; 33.73˚N, 72.93˚E during the non-breeding season i.e. July. After that, these animals were kept in separate cages in a semi-controlled environment in the Department's Primate Facility for a brief period. Four animals were long-term maintained under semi-controlled conditions in the Primate Facility. These animals were provided daily with fresh fruits, peanuts (0900-0930 h), boiled eggs (1100 h) and bread (1300-1330 h). Water was available *ad libitum*. All investigational procedures were approved by the Departmental Ethical Committee for Care and Use of Animals.

Mice. Ten adult male and ten adult female C57BL/6J mice (60-90 day of age) were used. All animals were maintained under 12 h light and 12 h dark cycle (lights on at 6.00 or 6.30 AM). All mice were provided with food and water *ad libitum*. All the experimental procedures were allowed by the Animal Welfare and Ethics Committee of the University of Otago, Dunedin, New Zealand.

Immunofluorescence

I. Tissue Collection

Hypothalamic blocks were obtained from nine adult male rhesus monkeys. In one experiment all animals were normally fed, while in another experiment two animals were normally fed and the two animals were 48 h fasted at the time of euthanasia. Three animals were sacrificed during the breeding season (October) and the two animals were sacrificed during non-breeding season (July). These animals were anaesthetized by ketamine hydrochloride (Ketamax, Trittau, Germany; 10-20 mg/kg BW, im). Hair of the head region were shaved with a razor, then skin area was thoroughly scrubbed with povidone/tincture of iodine and 70% ethanol and muscles were removed by using a scalpel. Then a sharp bone cutter was used to cut the skull bone in a circular manner from the top of head to expose the brain. Subsequent to the opening of the skull and exposure of the brain, 3-5 ml of sodium thiopental (30 mg/kg BW; Abbott Laboratories, Karachi, Pakistan) was injected intravenously. Brain was removed immediately (less than 1h after euthanasia) from the cranium and placed on a cold glass plate with ventral side up. Hypothalamic blocks including POA and MBH were dissected out from the brain by following the dissection procedure as described for rhesus monkey previously (Shibata et al., 2007). Briefly, taking median eminence as a central point, mammillary bodies (posteriorly) and

anterior commissure (anteriorly) as landmarks, one coronal cut was made rostral to the anterior commissure and another through the mammillary bodies. Then, two parasagittal cuts were made at about 5 mm on each side of midline. At the caudal boundary of the optic chiasm, a last cut dissected out square hypothalamus block. Further hemi-hypothalamic blocks including MBH and POA were made by a cut along the medial line. The blocks were bisected along the medial line. Hemi-hypothalamic blocks were fixed in a fixative for immunocytochemistry and other hemi-hypothalamic blocks were quickly immersed in liquid nitrogen. Then, hemi-hypothalamic blocks were stored at -80˚C until RNA extraction for real time PCR. To study testicular morphology, testicular tissues were obtained from one testicle of each animal. Blood samples were also drawn from these animals before euthanasia for hormone analysis.

All animals were sacrificed at day time (0010-0012) to make sure that all the experimental conditions were uniform for the study of the interaction of kisspeptin neurons with NR1 subunit of NMDA receptors and also for the quantification of *Kiss1*, *Kiss1r*, *NR1*, and *GAD67* mRNA levels in hypothalamus of adult male rhesus monkeys during breeding season, non-breeding season, fed and fasting conditions.

II. Tissue Fixation and Serial Sectioning

Hemi-hypothalamic blocks of all animals were fixed in 4% paraformaldehyde solution in 0.1M phosphate buffer saline (PBS), (pH 7.4) for 14-18 h at 4˚C. Then hypothalamic blocks were transferred to 20% sucrose solution in 0.1 M PBS for 3 days at 4˚C and then to 30 % sucrose solution at 4˚C until they sank. Consecutive 25 µm thick sections all over the whole hypothalamic block were cut on a cryostate (Bright OTF 5000; Bright Instrument Company, Huntingdon, UK) and stored in a

cryopreservative solution (1% polyvinylepyrrolidone, 30% sucrose and 30% ethylene glycol in PBS) at -20˚C until used for immunohistochemistry.

III. Fluorescence Immunocytochemistry

Free floating 25µm thick sections were processed for fluorescent immunocytochemistry to view the kisspeptin and NMDAR1 (here after called NR1) immunoreactivity in the monkey hypothalami. In total, four sections were selected from each animal. Three sections were treated with a primary antibody solution while one was primary antibody omitted control section. Sections were washed in 50 mM PBS (pH 7.3; 8×15 min) at room temperature. To block non-specific binding, sections were incubated in an incubation solution containing 10% normal donkey serum and 10% normal rabbit serum, on a shaker for one hour at room temperature. Sections were then incubated in a cocktail of primary antibodies (GQ2; 1:120,000; kindly donated by Dr. Stephen Bloom of Imperial College London, Hammersmith Hospital, London, UK and NR1; 1:250; purchased from Chemicon, Temecula, California, USA) diluted in an incubation solution containing 10% normal rabbit and 10% normal donkey serum for 48 h at 4˚C on a shaker. It was followed by 4 washes in 50 mM PBS, each for 10min at room temperature. To perceive immunofluorescence, sections were incubated in secondary antibodies CF 488 rabbit anti-sheep IgG (1:250, Biotium, Hayward, California, USA) and Cy3-conjugated AffiniPure donkey anti-mouse IgG (1:400, Chemicon) for 2 h (in the dark) at room temperature. Sections were then washed in PBS (4 x 5 min) and mounted on super frosted slides (Marienfeld GmbH, Lauda-Königshofen, Germany) and coverslips were placed using Immunomount (Thermo Electron Corporation, Pennsylvania, Pittsburgh, USA) as a mounting medium. Slides were left overnight at 4˚C for drying in dark and stored at 4˚C until fluorescent microscopy. To check the

specificity of GQ2 and NR1, primary antibodies were omitted from the control sections.

IV. Microscopy

Kisspeptin and NR1 immunoreactivity was examined by using an Olympus fluorescent microscope (Olympus BX51, Tokyo, Japan) and photographs were taken using a digital camera attached to the microscope. Whole MBH area was scanned in three random sections from each animal. Total number of kisspeptin cells in each section was counted and mean ± SEM number of kisspeptin cells was calculated for each animal. Kisspeptin cells expressing NR1 were determined based on the analysis of merge images. Such double-labeled cells appeared to express yellow immunoreactivity. As the three random sections were selected from each animal therefore the number was not constant.

SYBR Green qRT-PCR

I. RNA Extraction

Whole RNA was isolated from hypothalamic tissues of adult male rhesus monkey by using TRIzol Reagent (Molecular Research Centre, Cincinnati, OH, USA) according to manufacturer's instructions. Briefly, pre-weighed 100 mg of hypothalamic blocks were grinded using pestle and mortar and homogenized in 1ml TRIzol Reagent. Then, 200 µl chloroform was added to homogenized samples and vigorously vortexed for 30 sec. It was followed by the centrifugation at 12000 rpm for 15 min at 4 ºC. Upper transparent phase (0.5 ml) was separated and poured into a fresh tube subsequently, 0.5 ml of isopropanol was added. Samples were left for 10 min at room temperature to allow precipitation and then centrifuged at 8000 rpm for 8 min at 4 ºC. Upper transparent aqueous phase was removed and wasted. A white

pellet was clearly observed at the bottom of each tube. The pellet was washed with 75% ethanol then centrifuged at 7500 rpm for 5 min at 4 ºC. Pellets were air dried after the removal of ethanol and dissolved at room temperature in RNAase free water (Invitrogen, GIBCO, NY, USA).

II. RNA Quantification

RNA quantity was calculated by using Thermo Scientific Nanodrop 1000 spectrophotometer (Wilmington, DE, USA).

III. cDNA Synthesis

cDNA synthesis from RNA was carried out by using First Strand cDNA Synthesis Kit (Fermentas, Osaka, Japan) according to the protocol provided by the Supplier. The cDNA was synthesized by using random hexamer primers. Initially, the reaction mixture was incubated for 10 min at 25˚C. Reaction mixture was further incubated at 42˚C for 60 min. Reaction was then stopped by heating at 70˚C for 10 min. The cDNA samples of hypothalamic tissues were stored at -20˚C until analysis.

IV. Primers and Dilutions

To prepare stock solution of primers, each primer was dissolved in diethylpyrocarbonate (DEPC) treated water at a concentration of 1 µg/µl. Then primers were centrifuged at 13000 rpm for 30 seconds. The stock of primers was incubated at room temperature for 15 min. 20 µl of the primer stock solution and 80 µl of DEPC water were added to the labeled eppendorfs to prepare a working stock of primers.

V. Relative Quantitative Real-time PCR

The expression of *Kiss1*, *Kiss1r*, *NR1* and *GAD67* genes were evaluated by the use of Real-Time PCR. 25 µl reaction mixture was prepared by using 12.5 µl of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fischer Scientific, Osaka, Japan), 0.5 µl of forward primer and 0.5 µl of reverse primer and 10 µl of RNAse free water. The primer sequences were synthesized by Fermentas Thermo Fischer Scientific (Osaka, Japan). Sequences of all primers are as follows

Kiss1r F 5-AACTCGCTGGTCATCTACGTCAT-3,

R 5-TCTGTGGCCGCCAGGTT-3 (XM 001117198.1; product size 86 bp);

Kiss1 F 5-AGAAAAGGTGGCCTCTGTGGA-3,

R 5-AGGCTCTGCTCCCACGG-3 (XM 001098284.2; product size 87 bp);

GAD67 F 5-GAGGACCCCGGACAGTAGAGGC-3,

R 5-GATCTTGAGCCCCAGTTTTCTG-3 (XM 001082995.2; product size 181 bp);

NR1 (GRIN1) F 5-AGGAACCCCTCGGACAAGTT-3,

R 5-CGAGTCCCAGATGAAGGCA -3 (XM 001117773.2; product size 183 bp);

GAPDH F 5-AAGTACGATGACATCAAGAAG-3,

R 5-GAAGAGTGGGTGTCGCTGTTG-3 (NM 001195426.1; product size 119 bp). Thermocycler conditions were a hot start at 95˚C for 10 min followed by 40 cycles each at 95˚C for 15 sec followed by 60˚C for 30 sec and 72˚C for 30 sec. All reactions were run in duplicate and cycle threshold (Ct) was calculated by using software (Rotor gene 3000 Real time PCR machine, Corbett Research, Sydney, Australia). Comparative expression was calculated by using the relative cycle threshold (Ct) method. Every sample was normalized to endogenous housekeeping

gene glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) expression by using the 2 delta Ct method.

Plasma Testosterone Measurement

Plasma concentration of total testosterone of adult male rhesus macaque was measured by using two different assays during different reproductive seasons [(enzyme linked immunosorbent assay (ELISA)] and different metabolic states [(enzyme immunoassay (EIA)]. The detail of each testosterone assay is given below:

I Testosterone ELISA

Plasma concentration of total testosterone during breeding and non-breeding seasons of adult male rhesus macaques was measured by using a commercial human testosterone ELISA Kit (Astra Biotech GmbH, Germany). The assay was performed by following the procedure supplied with the Kit. Assay sensitivity was 0.2 nmol/L, and intra-assay coefficient of variation was less than 7%.

i. Principle of the Assay

The testosterone assay is a competitive type of assay which is based on the competition between testosterone in the samples, and testosterone conjugated with horseradish peroxidase (testosterone-HRP) for a limited number of testosterone antibodies fixed on microplate. After incubation, the bound/free testosterone separation was performed by washing of unbound testosterone-HRP by using distilled water. The HRP enzyme in the bound fraction reacts with tetramethylbenzidine (TMB) substrate. This reaction develops blue colour that turns into yellow, when stop solution is added. Testosterone concentration

in the sample negatively correlates with the intensity of the colour and it is measured through calibration curve.

ii. Assay Procedure

This assay was performed in a 96 micro well plate. Each well was specified for sample, standard or quality control. First of all 10 µl of standards, samples and quality controls were poured into specified wells. After that, 100 µl of testosterone-HRP conjugate and 50 µl of rabbit anti-testosterone reagent was poured into each well. Thorough mixing of plate for 30 sec on a shaker was followed by an incubation for 90 min at 37ºC in the incubator (Sakura Finetek Europe B., Alphen aan den Rijn / The Netherlands). The entire mixture was removed onto an absorbent paper. It was followed by the addition of 100 µl of TMB substrate and incubation at room temperature (18-25ºC) for 20 min. Then 100 µl of stop solution (H_2SO_4) was added to stop reaction. The micro plate was thoroughly mixed for 30 sec. When the entire blue colour turned into yellow colour, the absorbance was calculated at 450 nm on a microtiter plate reader (Model 680XR, BIO RAD, Tokyo, Japan) within 15-20 min.

iii. Result Calculation

Testosterone concentration in the samples was measured from the standard curve which was drawn by plotting the absorbance on y-axis and log of concentration of testosterone on x-axis. Plasma testosterone levels in the samples were estimated by point to point method of the Bio Rad microplate manager/PC software (V 5.2.1; Bio-Rad Laboratories, Hercules, CA, USA).

II Testosterone EIA

Plasma concentration of total testosterone of adult male rhesus macaques during normal fed and 48 h fasting states were measured by using a human testosterone

EIA kit (Amgenix, San Jose, USA). The assay was conducted by following the protocol and procedure provided with the kit. The sensitivity of the assay was 0.05 ng/ml.

i. Assay Principle

The assay is a competitive type, solid phase EIA. During incubation phase of the test, testosterone present in the sample and the testosterone which is labeled by HRP bind to the testosterone antibody, attached in solid phase to the wells. The remaining testosterone and HRP labeled testosterone is removed during washing. More bound conjugates inside the well indicate the lesser concentration of testosterone in sample and vice versa. After incubation with the TMB solution, colour is developed. The intensity of colour is inversely proportional to testosterone concentration in the sample. A standard curve is plotted to read testosterone concentration in the sample.

ii. Assay Procedure

First of all 10 µl of standards, specimens and controls was pipetted into selected wells carefully. 100 µl of testosterone-HRP conjugate reagent was then added into each well. After dispensing 50 µl of rabbit anti- testosterone reagent to each well, the microtiter plate was thoroughly mixed for 30 seconds and then incubated for 90 min at 37 ˚C. After incubation all the wells were washed 5 times with sterile distilled water. Next 100 µl of TMB substrate solution was added into each well. Gently mixed for 10 seconds and then incubation was done at room temperature (25 ˚C) for 20 min under dark. After this 100 µl of stop solution was added into each well and gently mixed for 30 seconds at room temperature. The OD was read at 450 nm by using a microtiter well reader (680XR, BIO RAD, Tokyo, Japan) within 15 min.

iii. Calculation of Results

A standard curve was plotted by taking log testosterone standard concentrations on X-axis and the relevant absorbance on Y-axis. The standard curve was fitted in a linear regression trend line by using software Microsoft Excel, version 2010 (Microsoft Excel software Inc., Albuquerque, New Mexico, USA). The linear regression estimation was used to calculate testosterone concentration in the plasma.

Blood Glucose Measurement

Blood glucose was measured by using Oncall Plus blood glucometer (ACON laboratories, San Diego, CA, USA).

I Assay Principle

The Oncall Plus blood glucometer uses an electrochemical enzymatic assay to quantify the glucose concentration in whole blood. The glucose test strips are used in the Oncall Plus blood glucometer, these strips have three components: an enzyme; glucose oxidase, a mediator; potassium ferricyanide and two electrodes. Glucose oxidase catalyzes a chemical reaction which converts glucose into gluconic acid. Then this gluconic acid reacts with the potassium ferricyanide and converts it into potassium ferrocyanide. This potassium ferrocyanide then reacts with the electrode metal and causes an electrical current. Glucometer is able to interpret this electrical current and uses this information to calculate how much glucose is present in the blood. This information then appears on the screen of glucometer.

II Assay Procedure

A quality control test was performed by using the glucose control solution of level 1 (82-135 mg/dL) provided with the glucometer to check the accuracy of glucometer

and test strips before starting the assay. A drop of blood was placed at the tip of glucose test strip. Blood was automatically seeped into the reaction cell. This was followed by a chemical reaction in the reaction cell. The glucometer detected an electrical current produced for a short time during the reaction. Then, the blood glucose concentration was measured based on that electrical current. The result was displayed on the glucometer screen.

Measurement of Testicular Volume

The volume of both right and left testis was measured by using formula $V= (\pi w^2 l)/6$, in which 'w' denotes width, 'l' represents the length of each testicle and V is volume in ml (Steiner and Bremner, 1981). Volumes of right and left testis were added to get the total volume of the testes.

Testicular Morphology

I. Tissue Processing

To examine the changes in testicular morphology during breeding and non-breeding season, testicular tissues were extracted and immediately placed in sera fixative (60 ml absolute alcohol (Sigma-Aldrich GmbH, Steinheim, Germany), 30 ml formaldehyde (Merck, Darmstadt, Germany) and 10ml of glacial acetic acid (Merck, Darmstadt, Germany) for 4-5 h. Tissues were then dehydrated in ascending grades of alcohol (Sigma-Aldrich) and placed in cedar wood oil (Serva Electrophoresis, GmbH, Heidelberg, Germany) until they became clear. The tissue was then given two washes in benzol (10 min each) and placed in benzol+paraplast (Fluka AG, Buchs, Switzerland) (1:1) for 20 min at 60˚C. Then tissues were given 3 paraplast washes (each for 12h at 60˚C). Tissues were then embedded in paraffin wax at 60˚C.

5µm thick sections were cut on a microtome (Thermo Electron Corporation, Cheshire, UK). Hematoxylin and Eosin staining was performed to stain cell structures such as cytoplasm, nucleus and cell organelles. Periodic acid-Schiff staining was done to stain spermatozoa, spermatids and basement membrane of seminiferous tubules.

II. Periodic acid-Schiff Staining

Before starting the staining procedure, testicular sections were fixed on glass slides. To deparaffinize, sections were given two xylol washes, each for five min. Sections were then rehydrated by passing through descending grades of alcohols (100%, 90% and 70% each for one minute) followed by a tap water wash for 1min. Then sections were treated with 0.5% periodic acid solution for 5 min. Then sections were given a fresh water wash for 2min. Then sections were treated with Schiff's reagent for 10-15 min. This was followed by a 5 min tap water wash that intensified the magenta colour. Then sections were counterstained with hematoxylin (BDH, England) for 2-4 min. After washing with tap water, sections were dehydrated and given two xylol washes each for 1min. The sections were then coverslipped with DPX (distyrene, a plasticizer, xylene) mounting media (BDH Chemicals, Poole, UK).

III. Hematoxylin and Eosin Staining

To deparaffinize, sections were given two xylol washes, each for five min. Sections were then rehydrated by passing through descending grades of alcohols (100%, 90% and 70% each for one minute). Then sections were placed in hematoxylin stain for 5 min. Sections were then given a fresh water wash for 2 min. Then sections were dipped 2-3 times in 1% acid alcohol. Following a 2 min wash with tap water, sections were placed in eosin (BDH, England) for 2 min. After washing with tap

water, sections were dehydrated and given two xylol washes each of 1min. The sections were then coverslipped with DPX mounting media.

Chromogen Immunocytochemistry

I Tissue Collection

All animals were deeply anaesthetized with an intraperitoneal (ip) injection of sodium pentobarbital (3 mg⁄100 µl), when the mouse had no toe pinch reflex it was placed in supine position. Skin was picked up with the forceps just below the sternum, a horizontal cut was made in the skin. Muscle wall was grasped over the end of the sternum with forceps, and lifted slightly. A cut was made through the muscle wall, and the incision was extended down to the profile of the ribs to expose diaphragm. The sternum was grasped with the forceps, snipped through the lateral portion of the ribs and diaphragm to deflate the lungs. The diaphragm was cut carefully away from the border of the ribs to gain access to the chest cavity. The ribs were snipped up bilaterally so that the rib plate was folded back towards the head. A small snip was made in the right atrium to allow the outflow of blood and paraformaldehyde. A 23G needle was inserted into the left ventricle and 15-20 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PBS; pH 7.6) was injected at a rate of 3-5 ml per minute.

II Tissue Fixation and Serial Sectioning

The brain was removed and placed in the 4% paraformaldehyde for 1h at 4˚C. Brain tissues were then moved to a 30% sucrose solution in tris buffer saline (TBS) for one night at 4˚C. Then, brains were placed on the stage of a sliding freezing microtome and three sets of 30µm thick coronal sections were cut from the forebrain to the brain stem.

III Immunocytochemistry

Free floating chromogen labeling was performed on one set of mouse brain sections. To block endogenous peroxidases, sections were placed in hydrogen peroxide solution (11.4ml TBS, 8 ml methanol, 600 µl H_2O_2 30%) for 10min. Then, sections were washed three times in TBS each for 10 min. After that, sections were incubated in rabbit polyclonal primary antisera directed against androgen receptors (AR20 gift from Gail Prins, University of Illinois, Chicago, IL, USA; 1:500) or polyclonal rabbit anti ERα (1:10,000; Millipore, Billerica, MA, USA) diluted in TBS containing 0.3% Triton X-100 and 0.25% BSA (hereafter called incubation solution, pH 7.6) and 2% normal goat serum for 48h at 4°C on orbital shaker. After subsequent washing in TBS (3x10), sections were incubated in peroxidase-conjugated anti-rabbit immunoglobulins (1:400; Vector Laboratories, Burlingame, CA, USA) for 4h at room temperature. Then, sections were washed in TBS (3 x 10min). Sections were then incubated in Vector Elite avidin–peroxidase (1:100; Vector Laboratories) for 90 min at room temperature. After three TBS washes sections were treated with glucose-oxidase and NiDAB (12.5 mg/ml) that produces black precipitates within the stained nucleus. Then, sections were given three TBS washes each for 10 min. For double immunolabeling, sections were exposed to 3% hydrogen peroxide to block endogenous peroxidases. Then three TBS washes each for 10 min. Then sections were incubated in polyclonal rabbit anti-kisspeptin antiserum (AC566, donated by Alain Caraty, Institut National de la Recherche Agronomique (INRA), Paris, France) at a dilution of 1:10,000 for 48h at 4°C. Sections were then incubated in biotinylated anti-rabbit immunoglobulins (1:200; Vector Laboratories) for 90min at room temperature. Sections were then treated with glucose-oxidase and DAB without nickel to reveal immunocytochemistry as a

brown precipitate within the cytoplasm. After this, sections were thoroughly washed in TBS and mounted on gelatin coated glass slides. Then the slides were air-dried and passed through ascending grades of alcohols. Then the slides were placed in xylene and cover slipped with DPX (BDH Laboratory Supplies, Poole, UK).

IV Microscopy

Sections were studied by using Olympus BX51 (Olympus, Tokyo, Japan) microscope under ordinary light. Kisspeptin neurons were stained brown (cytoplasm only) while androgen and estrogen receptors were stained black (nucleus). Single (only brown cytoplasm) and double labeled (brown cytoplasm with black nucleus) cells were counted and percentage of double labeled cells was calculated from these data.

Statistical Analyses

Statistical comparison of *Kiss1, Kiss1r, NR1* and *GAD67* mRNA contents and kisspeptin neurons expressed NR1 in the MBH and POA of adult male rhesus macaque (during different reproductive seasons and metabolic states) was made by unpaired student-t test. Comparison of blood glucose and plasma testosterone levels was done by unpaired student-t test. In mice study, single and double labeled kisspeptin neurons expressed AR and ERα were also analysed statistically by unpaired student-t test. All of the statistical analyses were done by using Graphpad Prism software version 6 (GraphPad Software. Inc., La Jolla, CA, USA) (www.graphpadprism.org). Data were presented as mean ± SEM and were considered statistically significant when $P<0.05$.

Chapter 1

Studies on the neurotransmitter regulation of kisspeptin neurons

Expression of kisspeptin (*Kiss1*), kisspeptin receptor (*Kiss1r*), NMDA receptor subunit (*NR1*) and GABA catalysing enzyme (*GAD67)* genes in the hypothalamus of male rhesus macaque: Correlative changes with seasonal breeding

ABSTRACT

Background: Hypothalamic kisspeptin signaling has been recently observed to correlate with seasonal breeding in free ranging male rhesus monkeys. Recent evidence has suggested contribution of excitatory amino acid neurotransmitters and inhibitory amino acid neurotransmitters in the regulation of kisspeptin neurons.

Objective: This study has focused on analysing the interplay of kisspeptin neurons with afferent excitatory (glutamate) and inhibitory (GABA) neurotransmitters in the brain of male monkeys during the breeding and non-breeding seasons where HPG-axis is altered. We hypothesized that kisspeptin stimulation may occur due to the increase in glutamate excitatory inputs and decrease in inhibitory GABA based cues during breeding season.

Materials and Methods: mRNA was extracted from the MBH and POA of five intact adult male rhesus monkeys (*Macaca mulatta*) captured from wild during the breeding (n=3; October; plasma T levels: 26.15 ± 2.64 nmol/L; TV: 69.00 ± 1.00 ml) and non-breeding (n=2; July; plasma T levels: 4.09 ± 1.64 nmol/L; TV: 12.88 ± 0.31 ml) seasons. Real time PCR was performed by using specific primers to quantify the levels of *Kiss1*, *Kiss1r*, *NR1* and *GAD67* relative to *GAPDH*. Immunofluorescence was also performed to examine the interaction of kisspeptin neurons with NR1 subunit of NMDA receptors in hypothalamus of adult male rhesus macaque during the breeding and non-breeding seasons.

Results: Significantly high ($p<0.05$) expression of *Kiss1*, *Kiss1r* and *NR1* mRNA levels and low ($p<0.05$) expression of *GAD67* mRNA levels in the hypothalamus were synchronized with the breeding season. The expression levels of *Kiss1*, *Kiss1r*

and *NR1* mRNA were significantly reduced ($p<0.05$) and the expression of *GAD67* mRNA levels were elevated ($p<0.05$) during the non-breeding season. The number of kisspeptin neurons was significantly ($p<0.05$) increased during the breeding season, and the percentage of kisspeptin neurons expressing NR1 was also significantly ($p<0.05$) increased during the breeding season.

Conclusion: Based on correlative gene expression changes in the adult male monkey hypothalamus, we suggest that increased kisspeptin signaling during the breeding season is entrained by an increase in glutamate excitatory inputs and decreased kisspeptin signaling during the non-breeding season is occasioned by an increase in GABA based inhibitory cues. We also suggest that glutamate and GABA operate in concert, with contrasting effects, to control kisspeptin signaling in rhesus macaque across the season, leading to yearly change in fertility.

INTRODUCTION

Reproductive activity in many species is seasonal, ensuing from yearly variations in GnRH secretion (Karsch et al., 1984). In ewe, the neural mechanisms that are responsible for transition of photoperiodic signal into a hormonal signal act in the MBH to regulate GnRH release (Malpaux et al., 1998). There is an annual cycle of sexual activity and testicular volume in free-ranging male rhesus macaque, reproductively active in winter months from September to January and then becoming reproductively quiescent in summer months (Sade, 1964; Zamboni et al., 1974). Although the neural pathways involved in regulating seasonal reproduction are defined, however, the mechanisms that link season and reproduction remain to be fully determined. Kisspeptin is a peptide encoded by the *Kiss1* gene (Lee et al., 1996), which acts through the activation of Kiss1r (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). It has been established that kisspeptin plays an imperative role in puberty onset and maintenance of HPG axis in humans (de Roux et al., 2003). A host of studies have now established that kisspeptin is a fundamental regulator of GnRH release and provides a common afferent conduit for regulation of GnRH neurons. GnRH neurons are direct target for kisspeptin as indicated by the presence of kisspeptin receptor on GnRH neurons in rats and mice (Han et al., 2005; Irwig et al., 2004). In rhesus macaque, kisspeptin neurons have been found to be present in the ARC of hypothalamus but not in POA (Ramaswamy et al., 2008; Shibata et al., 2007). Furthermore, in ewe (Smith et al., 2007) and hamsters (Greives et al., 2007; Revel et al., 2006), it has been proposed that *Kiss1* expression in the hypothalamus may be regulated as a function of seasonal reproduction.

Glutamate an excitatory neurotransmitter, is another important regulator of GnRH neuronal activity. Intravenous administration of NMDA can result in precocious puberty in immature rats (Urbanski and Ojeda, 1987) and activation of ionotropic glutamate receptors plays an important role in both surge (Ping et al., 1994) and pulse (Bourguignon et al., 1989) modes of GnRH secretion in rats. Administration of glutamate can enhance circulating LH levels and this effect is exerted centrally, since exposure of pituitary slices to glutamate or glutamate injection directly into the hypophysis do not alter LH release (Jennes et al., 2002). In mammals, NMDA receptor is the most important type of glutamate receptors that is necessary for the secretion of GnRH (Bourguignon et al., 1989; Plant et al., 1989; Claypool et al., 2000). Though NMDAR1 mRNA has been observed to be coexpressed by GnRH neurons (Ottem et al., 2002), lack of c-fos immunoreactivity in GnRH neurons subsequent to NMDA administration (Saitoh et al., 1991; Lee et al., 1993), indicates that GnRH neurons may be excited by glutamate through intermediate neuronal pathways. Recently, it has been shown that glutamate acts on GnRH nerve terminals in a kisspeptin dependent way to contribute in GnRH/LH release in male mouse (d'Anglemont de Tassigny et al., 2010).

GABA is a key inhibitory neurotransmitter in the hypothalamus as well as in central nervous system (Decavel and Van Den Pol, 1990). Leranth et al. (1985) demonstrated GAD positive axons forming synaptic contacts with GnRH positive neurons in rats. GnRH neurons express GABAA receptors (Petersen et al., 1993) and results of in vivo micro infusion experiments show that GABA inputs to the GnRH cell bodies put forth an important inhibitory influence on the secretory activity of GnRH neurons in rats (Herbison et al., 1991). A main site for the regulation of GABAergic system is the control of the synthesis and function of GABA synthesizing enzyme, GAD, which

converts glutamate to GABA in one step (Martin and Rimvall, 1993). In mammals, two isoforms of GAD exist called GAD67 and GAD65 according to their molecular weights of about 67 and 65kDa (Erlander and Tobin, 1991; Martin and Rimvall, 1993).

It has been recently found that the blockade of ionotropic glutamate receptors unpredictably lowered LH response to the administration of Kp-10, whereas stimulation of NMDA ionotropic receptors that stimulates LH and FSH secretion did not result in enhancement of LH peak responses to Kp-10. GABAA receptor stimulation inhibits Kp-10 induced LH levels, while their blockade increases basal LH levels and prolonged responses to Kp-10 when GABAA and GABAB receptor inhibitors are co-administered in male rats (García-Galiano et al., 2012).

In spite of the above information, it is plausible that, in physiological settings, kisspeptin may work together with many other neurotransmitters, hormones and neuropeptides which are known to regulate GnRH secretion. The detection of the imperative functions of kisspeptin to regulate GnRH secretion has actually compelled to reconsider the impacts and mechanism of action of other regulatory inputs on GnRH neurons (Roa et al., 2008). It is important to find that whether these regulatory factors act in concert with kisspeptin. Currently our comprehension of such interactions is still poor. Therefore, this study was designed to analyse the interplay of kisspeptin neurons with afferent excitatory (glutamate) and inhibitory (GABA) neurotransmitters in the brain of male monkeys during breeding and non breeding seasons where HPG-axis is overtly altered. We hypothesized that seasonal kisspeptin stimulation occurs due to increase in glutamate excitatory inputs and reduction in inhibitory GABA based cues during breeding season. Kisspeptin-glutamate signaling was tracked by performing dual label immunofluorescence for kisspeptin and NR1.

Kisspeptin, glutamate and GABA signaling were tracked by monitoring the expression of *Kiss1, Kiss1r, NR1* and *GAD67* genes, respectively.

If this is a case, one would predict that expression of kisspeptin peptide and NMDA receptor and *Kiss1*, *Kiss1r*, and *NR1* mRNA contents would be increased whereas *GAD67* gene expression would be decreased in the breeding season. Thus, main objective of this study was to find out whether there are any correlative seasonal changes in the expression of *Kiss1*, *Kiss1r*, *NR1* and *GAD67* genes in the hypothalamus of adult male monkey. In addition, the objective was to find out changes in the expression of kisspeptin peptide and NR1 in the hypothalamus of adult male rhesus macaques during the breeding and non-breeding season.

MATERIALS AND METHODS

Animals

In this study, five adult intact male rhesus monkeys (*Macaca mulatta*) weighing 7.2-13kg in body weight, were used. Three animals were captured from Ayubia National Park, Khyber Pakhtunkhwa, Pakistan; 33.86˚N, 73.13˚E during the breeding season (October) while the other two animals were taken captive from Margalla Hills National Park, Islamabad, Pakistan; 33.73˚N, 72.93˚E during the non-breeding season (July). The animals were captured using a dart gun with the permission of the respective wildlife officials. Each animal was kept in a separate cage, in a single room, in a semi-controlled environment in the Department of Animal Science's Primate Facility for a week. These animals were fed fresh fruits, peanuts (0900-0930h), hard boiled eggs (1100h) and bread (1300-1330h). Water was provided *ad libitum*. All investigational protocols were permitted by the Department of Animal Science's Committee for Care and Use of Animals.

Tissue Collection and Blood Sampling

Hypothalamic blocks were taken out from five adult male rhesus monkeys. All animals were normally fed at the time of euthanasia. Three animals were sacrificed during the breeding season (October) and the other two animals were sacrificed during the non-breeding season (July). Ketamine hydrochloride (Ketamax, Trittau, Germany; 10-20 mg/kg BW, im) was administered to sedate animals. After opening of skull, sodium thiopental (30 mg/kg BW; Abbott Laboratories, Karachi, Pakistan) was also injected intravenously. The brains were removed from the skull immediately. Hypothalamic blocks including POA and MBH were dissected out from the brain by

following the dissection procedure described for rhesus monkey previously (Shibata et al., 2007). Briefly, taking median eminence as a central point, mammillary bodies (posteriorly) and anterior commissure (anteriorly) as landmarks, one coronal cut was made rostral to the anterior commissure and the second cut was made through the mammillary bodies. Then, two parasagittal cuts were made at about 5 mm on both sides of midline. At the caudal boundary of optic chiasm, a last cut dissected out square hypothalamic blocks. Further hemi-hypothalamic blocks including MBH and POA were made by a cut along the medial line. Hemi-hypothalamic blocks were placed in a fixative for immunocytochemistry and other hemi-hypothalamus was quickly immersed in liquid nitrogen and then placed at -80˚C until RNA isolation. Testicular dimensions were recorded from animals before euthanasia for calculation of testicular volume. To study testicular morphology, testicular tissue was obtained from one testicle of each animal. Blood samples were also drawn from all animals for hormone analysis. All animals were sacrificed at daytime (0010-0012) to make sure that all the experimental conditions were uniform.

Immunocytochemistry

Hemi-hypothalamic blocks of all animals were fixed in 4% paraformaldehyde solution in 0.1M PBS (pH 7.4) for 14-18 h at 4˚C. Then hypothalamic blocks were transferred to 20% sucrose solution for 3 days at 4˚C and then to 30% sucrose solution at 4˚C until they sank. Consecutive 25 µm thick sections all over the whole hypothalamic block were cut on a cryostate (Bright OTF 5000; Bright Instrument Company, Huntingdon, UK) and stored in a cryopreservative solution (1% polyvinylepyrrolidone, 30% sucrose and 30% ethylene glycol in PBS) at -20˚C until used for immunohistochemistry.

Four free-floating sections from each animal were processed for immunofluorescence to view the kisspeptin and NR1 immunoreactivity in the monkey hemi-hypothalamus. One section from each animal was also processed as a primary antibody omitted control. Sections were washed in 50 mM PBS (pH 7.3; 8×15min) at room temperature. Then, sections were incubated in incubation solution containing 10% normal donkey serum and 10% normal rabbit serum to block non-specific binding, on a shaker for one hour at room temperature. Sections were then incubated in a cocktail of primary antibodies (GQ2; 1:120,000; kindly donated by Dr. Stephen Bloom of Imperial College London, Hammersmith Hospital, London, UK and NR1; 1:250; purchased from Chemicon, Temecula, California, USA) diluted in incubation solution containing 10% normal rabbit and 10% normal donkey serum for 48 hr at 4˚C on a shaker. It was followed by four washes in 50 mM PBS, each for 10 min at room temperature. To perceive immunofluorescence, sections were incubated in secondary antibodies CF-488 rabbit anti-sheep IgG (1:250, Biotium, Hayward, California, USA) and Cy3-conjugated AffiniPure donkey anti-mouse IgG (1:400, Chemicon) to identify kisspeptin and NR1 immunoreactivity, respectively, for 2 h in the dark at room temperature. Then the sections were washed in PBS (4 x 5 min) and mounted on super frosted slides (Marienfeld GmBH, Lauda-Königshofen, Germany) and coverslips were placed using Immunomount (Thermo Electron Corporation, Pittsburgh, USA) as a mounting medium. After mounting, slides were left overnight in the dark at 4˚C for drying and stored at 4˚C until microscopy. To check the specificity of GQ2 and NR1, primary antibodies were omitted from the control sections.

Microscopy

Kisspeptin and NR1 immunoreactivity was viewed using Olympus fluorescent microscope (Olympus BX51, Tokyo, Japan) and photographs were taken using a

digital camera attached to the microscope. From each animal, four random sections containing MBH were selected for the analysis. Three sections were treated with antibody and one was primary antibody omitted control. Kisspeptin cells were stained green and NR1 expressing elements appeared as red. Whole MBH area was scanned in three random sections from each animal. Total number of kisspeptin cells in each section were counted and mean ± SEM number of kisspeptin cells was calculated for each animal. Kisspeptin cells expressing NR1 were determined based on the analysis of merge images. Such double-labeled cells appeared to express yellow immunoreactivity. There was variation in the number of kisspeptin neurons among three random sections and among the animals. Therefore, means were taken for number of kisspeptin neurons/section during the two seasons. Due to variation in section and animal, these neuronal numbers were different and could not be kept constant.

SYBR Green qRT-PCR

I Isolation of RNA and cDNA Synthesis

Whole mRNA was removed from hemi-hypothalamic block of breeding and non-breeding season adult male rhesus monkey with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) by using provider's protocol. RNA quantity was measured by using a Thermo Scientific Nanodrop 1000 spectrophotometer (Wilmington, DE, USA). First Strand cDNA Synthesis Kit (Fermentas, Osaka, Japan) was used to synthesize cDNA from RNA according to the protocol provided by the supplier with the random hexamer primers. Briefly, the process involved initial incubation for 10 min at 25˚C, then further incubation at 42˚C for 60 min followed by termination for 10 min at 70˚C. The cDNA samples were placed at -20˚C until further analysis.

II Relative Quantitative Real-time PCR

Expression of *Kiss1*, *Kiss1r*, *NR1* and *GAD67* genes were evaluated by the use of real-time PCR. The reactions were done by using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fischer Scientific, Osaka, Japan). Each reaction of 25 µl included 12.5 µl of SYBR Green, 0.5 µl of each primer and 10 µl of PCR water. The primer sequences were synthesized by Fermentas Thermo Fischer Scientific (Osaka, Japan). Sequences of all used primers are as follows, *Kiss1r* F 5-AACTCGCTGGTCATCTACGTCAT-3, R 5-TCTGTGGCCGCCAGGTT-3 (XM 001117198.1; product size 86 bp); *Kiss1* F 5-AGAAAAGGTGGCCTCTGTGGA-3, R 5-AGGCTCTGCTCCCACGG-3 (XM 001098284.2; product size 87 bp); *GAD67* F 5-GAGGACCCCGGACAGTAGAGGC-3, R 5-GATCTTGAGCCCCAGTTTTCTG-3 (XM 001082995.2; product size 181 bp); *NR1* (GRIN1) F 5-AGGAACCCCTCGGACAAGTT-3, R 5-CGAGTCCCAGATGAAGGCA -3 (XM 001117773.2; product size 183 bp); *GAPDH* F 5-AAGTACGATGACATCAAGAAG-3, R 5-GAAGAGTGGGTGTCGCTGTTG-3 (NM 001195426.1; product size 119 bp). PCR efficiencies were >97% for all genes and were calculated from standard curves. Each cDNA sample was run in duplicate. Thermocycler conditions were first a hot start at 95˚C for 10 min then 40 cycles each at 95˚C for 15 sec followed by 60˚C for 30 sec and 72˚C for 30 sec. All reactions were run in duplicate and cycle threshold (Ct) was calculated by using software (Rotor gene 3000 Real time PCR machine, Corbett Research, Australia). Comparative expression was calculated by using the relative cycle threshold (Ct) method. Every sample was normalized to endogenous housekeeping gene *GAPDH* expression by using the 2 delta Ct method.

Plasma Testosterone Measurement

Plasma concentration of total testosterone was measured by using a human testosterone ELISA Kit (Astra Biotech GmbH, Germany). Assay sensitivity was 0.2 nmol/L, and intra-assay coefficient of variation was less than 7%.

Measurement of Testicular Volume

Testicular volume was measured by using formula $V= (\pi w^2 l)/6$, where 'l' represents the length and 'w' denotes width of each testicle. Volumes of right and left testis were added to get the total volume of the testes (Steiner and Bremner, 1981).

Testicular Morphology

To examine the changes in testicular morphology during breeding and non-breeding seasons, testicular tissues were extracted at euthanization and immediately placed in sera fixative (60 ml absolute alcohol, 30 ml formaldehyde and 10 ml of glacial acetic acid) for 4-5 hours. Tissues were then dehydrated in increasing alcohol grades, and placed in cedar wood oil until they become clear. Then following two washes in benzol (10 min each), tissues were placed in benzol+paraplast (1:1) for 20 min. The tissues were then given 3 paraplast washes (each for 12 h at 60˚C). Tissues were then embedded in paraffin wax at 60˚C. Testicular blocks were cut on a microtome at 5µm thickness and stained with periodic acid schiffs and eosin and hematoxylin staining techniques, then mounted on glass slides and cover-slipped by using DPX (BDH Chemicals, Poole, England, UK) mounting media.

Statistical Analyses

Data are presented as mean ± SEM. Mean values in the breeding and non-breeding seasons were compared by applying the student-t test. Differences were measured to

be statistically significant when $P<0.05$.

RESULTS

Testicular and hypothalamic tissues were taken from five adult male rhesus monkeys during breeding and non-breeding season. Statistically significant ($P<0.05$) rise in basal plasma testosterone levels (Figure 1.1A) and in total testicular volume (Figure 1.1B) was observed during breeding season when compared with the respective values in the non-breeding season. Active spermatogenesis, representing all types of spermatogenic cells (spermatogonia, spermatocytes and elongated spermatids) was observed in the testis during the breeding season (Figure 1.1 C). While, testicular regression was observed during the non-breeding season with the depletion of germ cells in the lumen of the seminiferous tubules (Figure 1.1 D). Correlative changes in the *Kiss1*, *Kiss1r*, *NR1* and *GAD67* mRNA contents in the hypothalamus of male rhesus monkey during the breeding and non-breeding seasons are shown in Figures 1.2-1.5. Significantly high ($p<0.05$) *Kiss1*, *Kiss1r* and *NR1* mRNA levels and low ($p<0.05$) *GAD67* mRNA levels in the hypothalamus show synchrony with the breeding season. Expression of *Kiss1*, *Kiss1r* and *NR1* mRNA levels were significantly reduced ($p<0.05$) and the expression of *GAD67* mRNA was significantly elevated ($p<0.05$) during the non-breeding season as compared to the breeding season. Individual and mean numbers of single and double-labeled kisspeptin neurons are shown in Tables 1.1 and 1.2. Dual label immunofluorescence of kisspeptin and NR1 in hypothalamus of adult male rhesus monkey during the breeding and non-breeding season is shown in Figures 1.6-1.7. Kisspeptin peptide expression was significantly ($p<0.05$) higher during the breeding season (Figure 1.8A). Percentage of kisspeptin neurons with NR1 significantly ($p<0.05$) increased during the breeding season (Figure 1.8B) as compared to the non-breeding season.

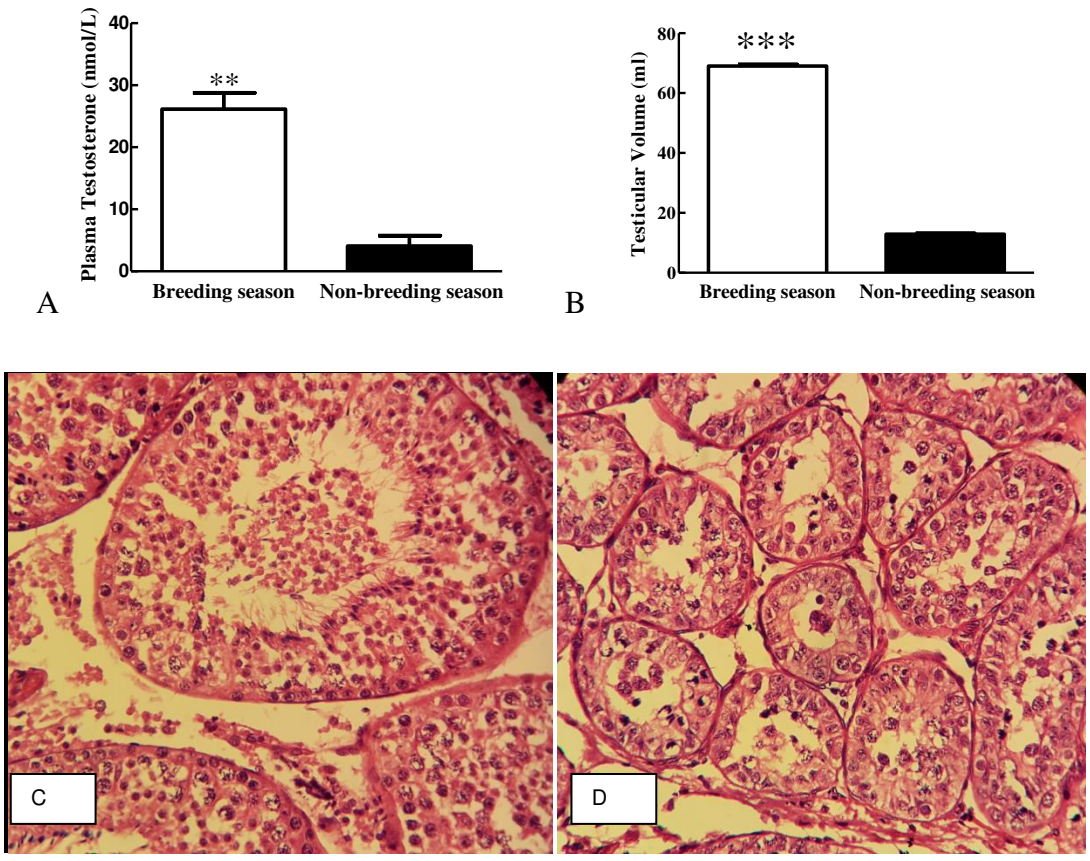

Figure 1.1. Comparison of mean (±SEM) basal plasma testosterone levels (**A**), mean (±SEM) total testicular volume (**B**) during the breeding (October; n=3) and non-breeding season (July; n=2) in adult male rhesus macaque. Basal plasma testosterone levels (**p<0.01) and testicular volume (***p<0.001) were significantly increased during breeding season as compared to non-breeding season. Cross section of testicular tissues of adult male rhesus macaque showing normal spermatogenesis during breeding season (C) and regression in spermatogenesis during non-breeding season (D). 400x magnification.

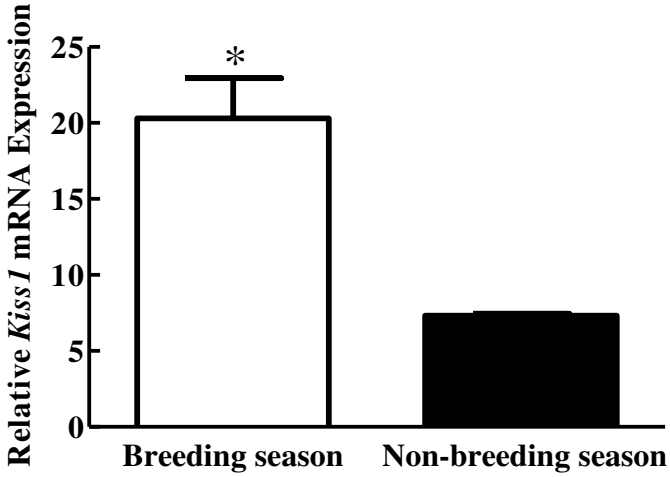

Figure 1.2. Mean (±SEM) relative *Kiss1* mRNA levels in the hypothalamus during breeding (October; n=3) and non-breeding season (July; n=2) in adult male rhesus monkeys. *Kiss1* mRNA contents were significantly (*P<0.05) increased during the breeding season as compared to non-breeding season. *GAPDH* was used as a housekeeping gene to normalize the *Kiss1* mRNA contents.

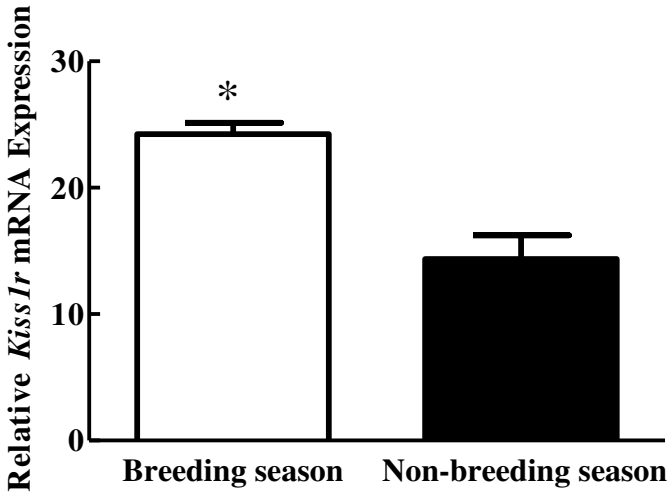

Figure 1.3. Mean (±SEM) relative *Kiss1r* mRNA expression during breeding (October; n=3) and non-breeding season (July; n=2) in adult male rhesus monkeys. *Kiss1r* mRNA contents were significantly (*P<0.05) increased during the breeding season as compared to non-breeding season. *GAPDH* was used as a housekeeping gene to normalize the *Kiss1r* mRNA contents.

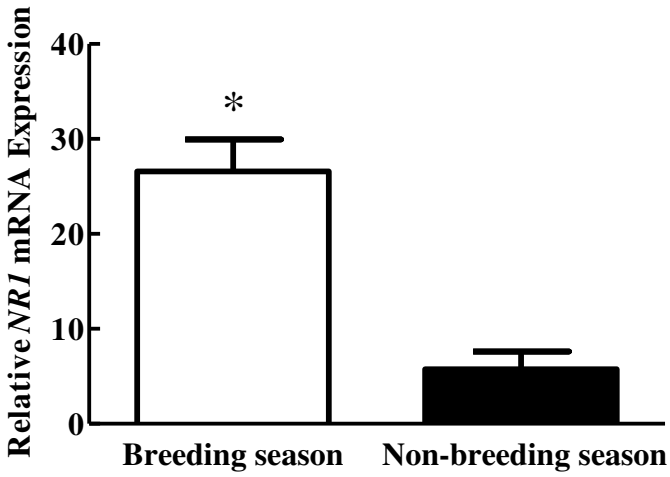

Figure 1.4. Mean (±SEM) relative *NR1* mRNA expression during breeding (October; n=3) and non- breeding season (July; n=2) in adult male rhesus monkeys. *NR1* mRNA contents were significantly (*P<0.05) increased during the breeding season as compared to non-breeding season. *GAPDH* was used as a housekeeping gene to normalize the *NR1* mRNA contents.

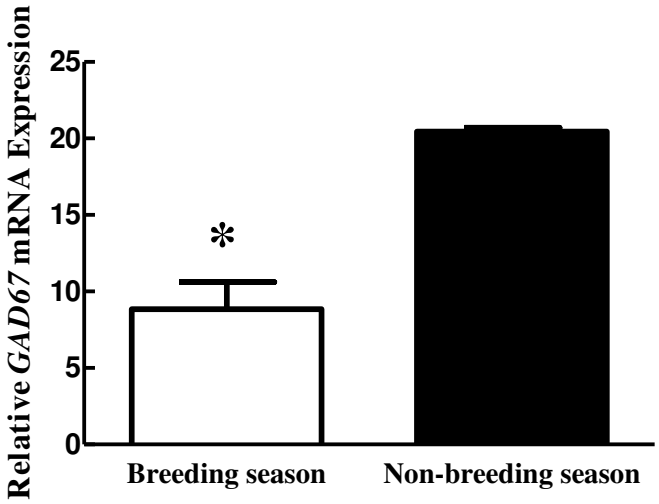

Figure 1.5. Mean (±SEM) relative *GAD67* mRNA expression during breeding (October; n=3) and non- breeding season (July; n=2) in adult male rhesus monkeys. *GAD67* mRNA contents were significantly (*P<0.05) increased during the breeding season as compared to non-breeding season. *GAPDH* was used as a housekeeping gene to normalize the *GAD67* mRNA contents.

Table 1.1. Comparison of individual and mean number of single (kp) and double labeled (dbl) kisspeptin neurons expressing NR1 in the MBH of three sections in adult male rhesus macaque (n=3) during the breeding season

Animal No.	Number of kp neurons during breeding season						Mean Kp	Mean dbl	% dbl
	Section 1		Section 2		Section 3				
	Kp	dbl	Kp	dbl	Kp	dbl			
M11	280	74	255	89	270	52	266.00	71.67	26.94
M12	260	81	259	54	240	89	263.00	74.67	28.39
M13	180	56	200	71	243	79	207.00	68.67	33.17
Mean							245.33	71.67	29.50
±SEM							±19.19		±1.882

Table 1.2. Comparison of individual and mean number of single (kp) and double labeled (dbl) kisspeptin neurons expressing NR1 in the MBH of three sections in adult male rhesus macaque(n=2) during non-breeding season

Animal No.	Number of kp neurons during non-breeding season						Mean Kp	Mean dbl	% dbl
	Section 1		Section 2		Section 3				
	Kp	dbl	Kp	dbl	Kp	dbl			
M14	158	23	122	17	70	27	116.67	22.33	19.14
M15	132	16	179	13	100	30	137.00	19.67	14.35
Mean							126.83	21.00	16.75
±SEM							± 10.17		±2.39

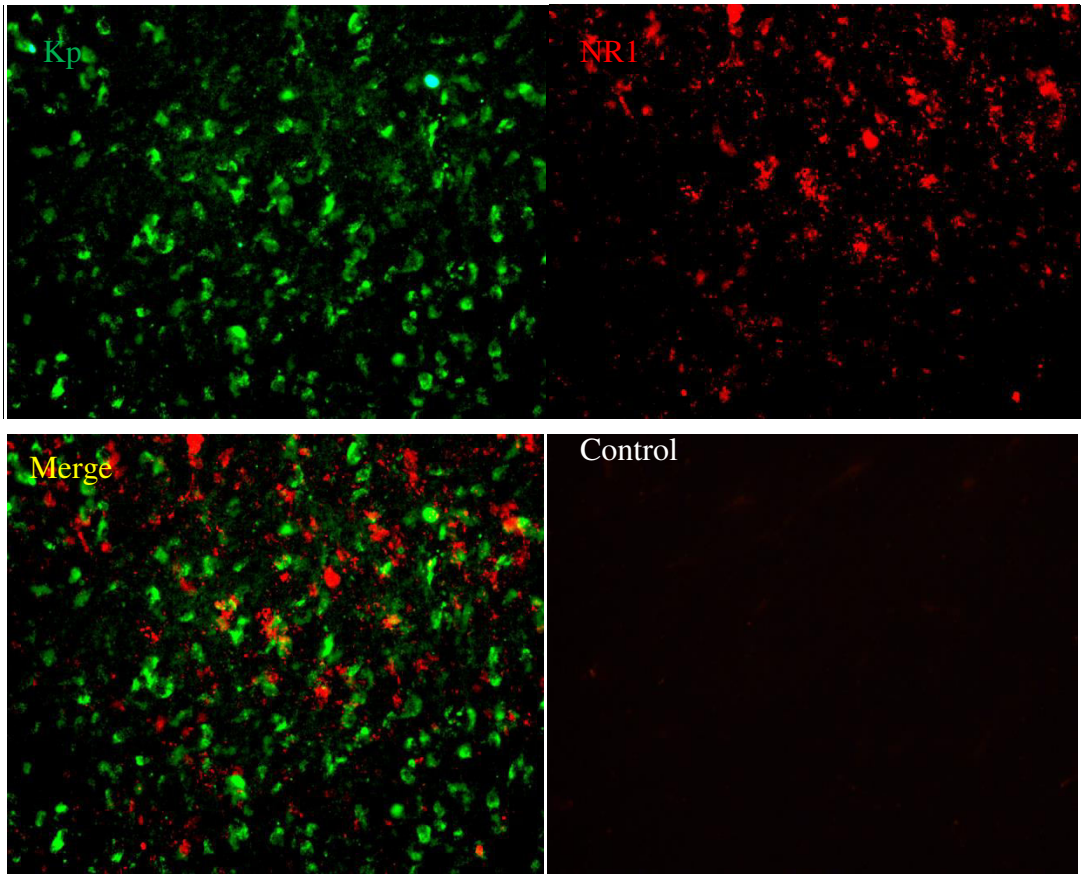

Figure 1.6. Fluorescent microscopic images showing kisspeptin immunoreactivity in green (upper left) and NR1 subunit of NMDA receptor in red (upper right) in the MBH of male rhesus macaque during breeding season. Merge image of kisspeptin and NR1 (lower left) showing co-localization (yellow colour) and primary antibody omitted control (lower right). Photographs were taken at 200x magnification.

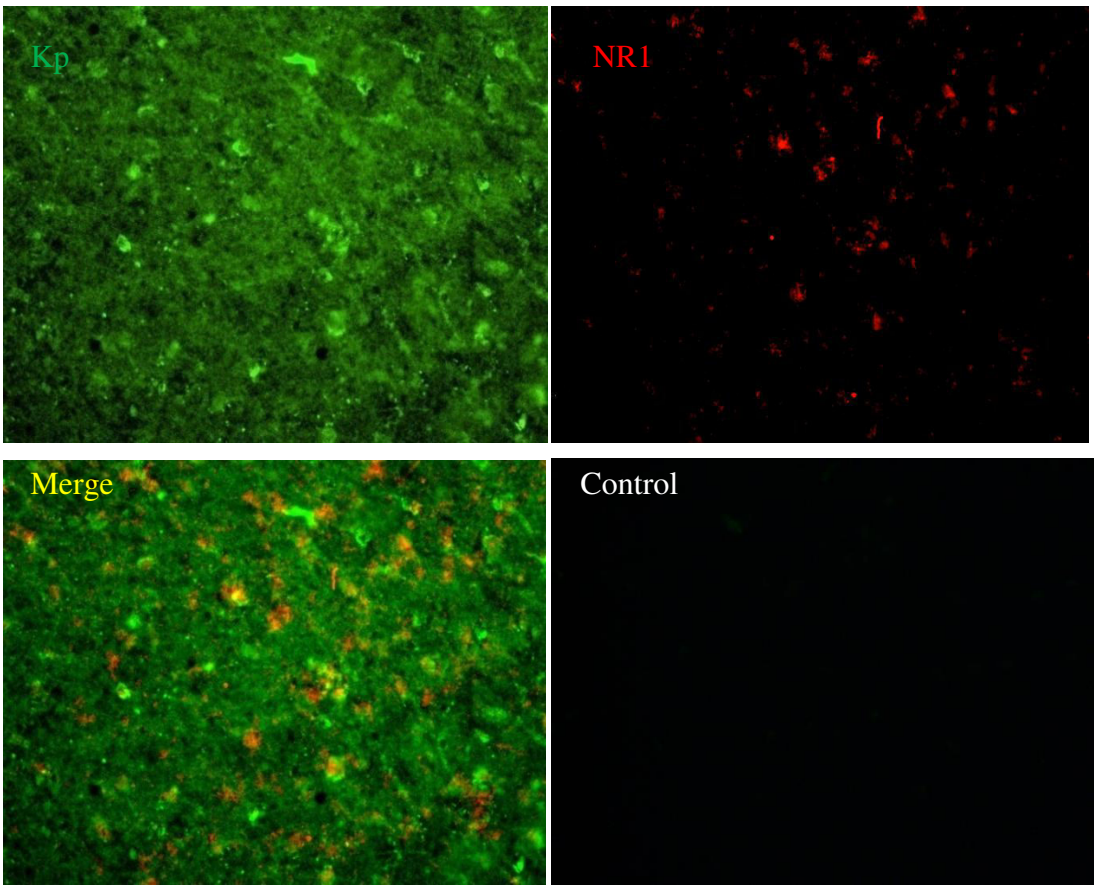

Figure 1.7. Fluorescent microscopic images showing kisspeptin immunoreactivity in green (upper left) and NR1 subunit of NMDA receptor in red (upper right) in the MBH of male rhesus macaque during non-breeding season. Merge image of kisspeptin and NR1 (lower left) showing co-localization (yellow colour) and primary antibody omitted control (lower right). Photographs were taken at 200x magnification.

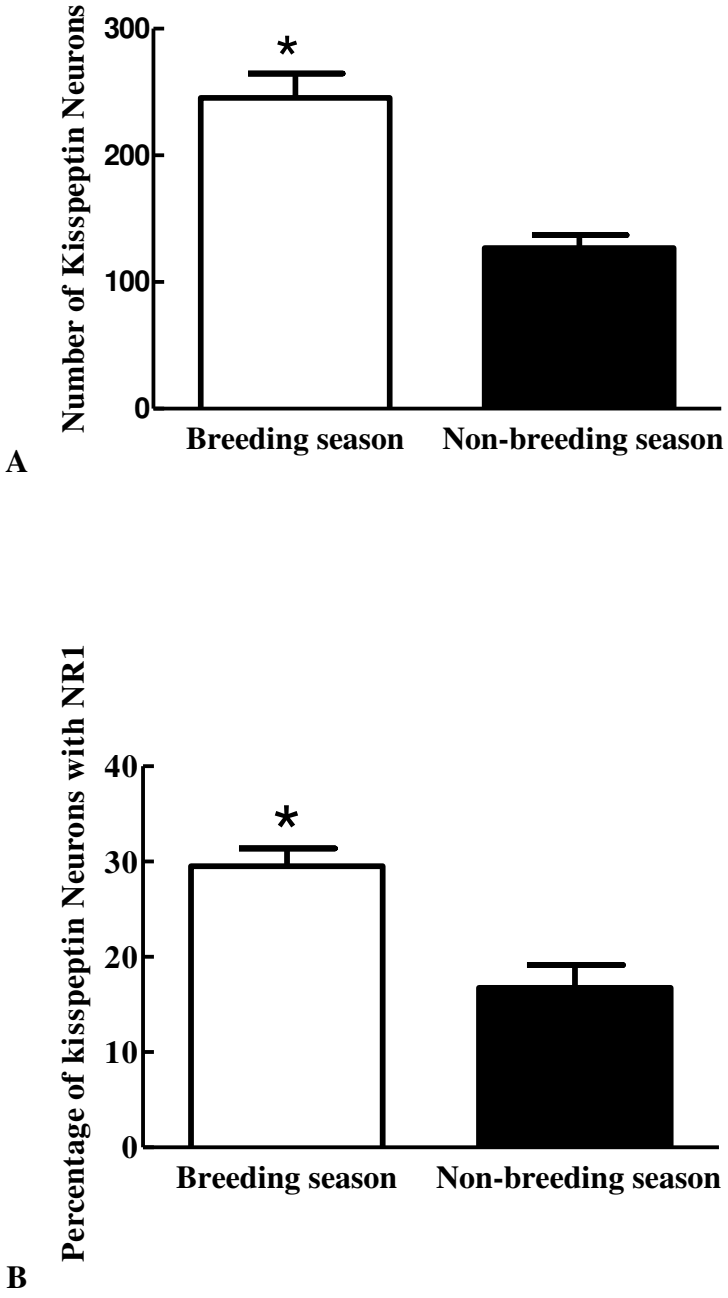

Figure 1.8. Comparison of the number of kisspeptin neurons (**A**) and percentage of kisspeptin neurons expressing NR1 (**B**) in MBH of adult male rhesus monkeys during breeding (n=3) and non-breeding (n=2) seasons. Number of kisspeptin neurons (*$p<0.05$) and percentage of kisspeptin neurons expressing NR1 (*$p<0.05$) significantly increased during breeding season.

DISCUSSION

Our results provided strong evidence that season related changes in HPG axis involve increased hypothalamic kisspeptin expression during the breeding season and decreased kisspeptin expression during the non-breeding season in the free ranging adult male monkeys. In the present study elevated *Kiss1* and *Kiss1r* expression was evident in the breeding season. However, the actual mechanisms involved in this stimulation of kisspeptin signaling pathway remain to be elucidated. Therefore, the present study has focused on analyzing the interplay of kisspeptin with other excitatory and inhibitory neurotransmitters during the breeding and the non-breeding seasons.

In this study seasonality was clearly evident in the free ranging adult male monkeys as basal plasma testosterone levels and testicular volume were elevated during breeding season as compared to non-breeding season. Our present results show that hypothalamic *Kiss1*, *Kiss1r* and *NR1* mRNA levels were significantly higher while *GAD67* mRNA levels were significantly lower in the breeding season as compared to the non-breeding season. Our results showed an increase in kisspeptin peptide expression and the percentage of kisspeptin neurons expressing NR1 during the breeding season. These findings are in line with the previous studies showing enhancement of glutamatergic signaling and reduction in GABAergic signaling during the breeding season as compared to the non-breeding season in ewe (Sergeeva et al., 2009). The increase in *NR1* mRNA contents and NR1 expression and decrease in *GAD67* mRNA expression might be involved in the activation of the monkey hypothalamic kisspeptin signaling during the breeding season.

There is an evidence that kisspeptin interacts with both glutamate and GABA. This point of view is supported by the observation that in the ARC of mice an NMDA receptor agonist administration, causes an increase of c-fos expression in kisspeptin cells, thus suggesting the regulation of kisspeptin neurons by glutamate (d'Anglemont de Tassigny et al., 2010). Similarly, an interaction of kisspeptin with GABA has been shown. This evidence is supported by the observation that in prepubertal monkeys, bicuculline, GABA antagonist administration, arouses kisspeptin release (Kurian et al., 2012).

That the kisspeptin signaling is strongly modulated by excitatory and inhibitory neurotransmitters, is further supported by the observation of activation of ARC-KNDy neurons (Cheng et al., 2010) by NMDA and inhibition of KNDy neurons by GABA (Gottsch et al., 2011; Qiu et al., 2011; Kurian et al., 2012). Similar to the ARC-KNDy cells, RP3V-Kiss1 neurons are also strongly activated by glutamate and inhibited by GABA (Ducret et al., 2010).

Similar to our findings of breeding season related modulation of *Kiss1* expression in the monkey hypothalamus, the *Kiss1* mRNA expression is also increased during short days in short day breeders such as ewe (Smith et al., 2007) whereas in long day breeders such as Syrian hamsters *Kiss1* mRNA levels in AVPV are increased during long days (Paul et al., 2009). In the same way, *Kiss1* mRNA levels in the ARC of male Syrian hamsters are decreased when they are shifted to short day environment from long day conditions (Revel et al., 2006). Variations in *Kiss1* mRNA levels during different seasons seem to be dependent on melatonin, as reduction in kisspeptin expression after short day treatment is inhibited by pineal gland ablation (Revel et al., 2006). However, kisspeptin neurons do not express melatonin receptors in ewe that shows a possible indirect effect of melatonin on kisspeptin neurons (Li et

al., 2011). Melatonin on the other hand, may interact with glutamate and GABA (Marquez de Prado et al., 2000). Furthermore, melatonin receptor (MT1) has also been co-localized with glutamatergic neurons in dorsal habenula neurons of transgenic mouse (Evely et al., 2013). GABAergic neurons localized in retina also express MT1 receptors in Guinea pig (Fujieda et al., 2000). On the basis of these findings, we suggest that melatonin cues differently regulate glutamate and GABA signaling in the primate hypothalamus which in turn modulates kisspeptin signaling.

In summary, present results suggest that increased kisspeptin signaling during breeding season is due to increased glutamate action and decreased GABA levels in the monkey hypothalamus. Restoration of kisspeptin expression during breeding season may be associated with an elevation of glutamate activity as indicated by an increase in *NR1* mRNA content and NR1 protein expression in male monkeys, and decreased GABA signaling as shown by lowered expression levels of GABA synthesizing enzyme gene. On the basis of changes in the pattern of gene and protein expression during the breeding and non-breeding season in adult male monkey hypothalamus, we suggest that increased kisspeptin signaling during the breeding season is due to increase in glutamate excitatory inputs and decreased kisspeptin signaling during the non-breeding season is a result of an increase in GABAergic inhibitory signals. We also propose that glutamate and GABA operate in concert, with complementary effects, to control kisspeptin signaling in rhesus macaque across the season, leading to yearly change in fertility.

Chapter 2

Studies on the neurotransmitter regulation of kisspeptin neurons

Changes in kisspeptin (*Kiss1*), kisspeptin receptor (*Kiss1r*), NMDA receptor subunit 1 (*NR1*) and GABA synthesizing enzyme (*GAD67)* genes expression in the hypothalamus of adult male rhesus macaque (*Macaca mulatta*) during fed and short term fasting: a potential mechanism for the attenuated kisspeptin signaling during short term fasting

ABSTRACT

Background: HPG axis is suppressed by short term fasting. Recently it has been found that kisspeptin signaling also plays an important role in metabolic regulation of the HPG axis. It has been reported that hypothalamic *Kiss1* and *Kiss1r* mRNA expression decreases after short term fasting in male rhesus monkey. But the mechanism involved in the reduction of kisspeptin signaling during short term fasting is unknown. Recent studies have suggested the role of afferent excitatory and inhibitory neurotransmitters in the regulation of kisspeptin neurons.

Objective: This study was designed to examine the regulation of kisspeptin neurons by excitatory (glutamate) and inhibitory (GABA) neurotransmitters in the hypothalamus of adult male rhesus monkey during fed and 48 h fasting states. We hypothesized that decreased kisspeptin signaling after 48 h of fasting may be due to decrease in excitatory inputs and increase in inhibitory inputs to kisspeptin neurons.

Materials and Methods: mRNA was extracted from hemi-hypothalamic blocks of four adult male rhesus monkeys (*Macaca mulatta*). Two of these animals were subjected to food deprivation for 48 h but the water was freely available while the other two were fed *ad libitum*. Quantity of *Kiss1*, *Kiss1r, NR1* and *GAD67* mRNA was measured by SYBR green RT-PCR. Blood samples were also drawn from these animals to measure glucose and testosterone levels. Immunofluorescence was performed to examine the interaction of kisspeptin neurons with NR1 subunit of NMDA receptors in hypothalamic MBH of adult male rhesus macaque during fed and 48 h fasted conditions.

Results: Plasma testosterone ($p<0.05$) and blood glucose ($p<0.001$) levels were significantly decreased after short term fasting. Hypothalamic *Kiss1, Kiss1r* and *NR1* mRNA expression levels were significantly ($p<0.05$) reduced in adult male rhesus monkeys which were fasted for 48 h as compared to those which were fed *ad libitum*. There was no clear difference in *GAD67* mRNA expression levels between the two groups. The number of kisspeptin neurons was significantly ($p<0.05$) decreased after 48 h fasting, and the interactions of kisspeptin neurons with NR1 expressing elements were also significantly ($p<0.05$) reduced after 48 h fasting.

Conclusion: Our results suggest that decreased kisspeptin signaling during fasting occur due to reduction in glutamatergic inputs to kisspeptin neurons. Further it suggests that fasting induced suppression of kisspeptin signaling is not mediated through GABAergic neurons and GABA is not involved in fasting induced suppression of the primate HPG axis.

INTRODUCTION

Reproduction is one of the most energy demanding processes and a strong connection exists between reproduction and metabolic status of the individual. Metabolic information from the peripheral tissues and environment is translated into hormonal signals that act on the central regulators of reproduction. Sufficient amount of energy is critical for the onset of puberty and maintenance of fertility in adulthood (Roa et al., 2010). An excess amount of energy (in case of obesity) and energy insufficiency (in case of excessive exercise, anorexia nervosa and diabetes) lead to the failure in puberty onset and hypogonadotropic hypogonadism in adulthood (Genazzani et al., 2010; Bruni et al., 2011). HPG axis is very sensitive to the metabolic fuels of the individual (Wade et al., 1996; Schneider, 2004; Wade and Jones, 2004). Fasting suppresses HPG axis and impairs reproduction (Kuderling et al., 1984; Badger et al., 1985; Cameron and Nosbisch 1991). A large amount of data has shown that fasting induced suppression of reproduction is due to a decrease in GnRH release but not due to the inhibition of HPG axis sensitivity to GnRH (Bergendahl et al., 1991; Cameron and Nosbisch 1991; Aloi et al., 1997).

On the basis of many genetic and pharmacological studies, it has been well established that kisspeptin regulates GnRH release (Gottsch et al., 2006; Plant, 2006; Tena-Sempere, 2006). It is not only involved in the pubertal increase in GnRH secretion (Han et al., 2005; Shahab et al., 2005), but also plays an important role in the maintenance of GnRH secretion in adulthood (Dhillo et al., 2005). Recently, it has been found that kisspeptin mediates metabolic cues to the HPG axis. Reduction in metabolic fuels, during lactation and fasting, reduces the levels of hypothalamic *Kiss1*

mRNA (Castellano et al., 2005; Luque et al., 2007; Yamada et al., 2007) as well as *Kiss1r* mRNA in rodents (Luque et al., 2007) and primates (Wahab et al., 2011).

Glutamate and GABA are important excitatory and inhibitory neurotransmitters in the CNS. Kisspeptin neurons interact with GABA (Kurian et al., 2012) and glutamate (d'Anglemont de Tassigny et al., 2010). In the ARC of mice, NMDA receptor agonist administration, causes an increase in c-fos expression in kisspeptin cells, thus suggesting the regulation of kisspeptin neurons by NMDA (d'Anglemont de Tassigny et al., 2010). EAA are also sensitive to metabolic fuel (Bucholtz et al., 1996). EAA stimulate apparent inactive GnRH neurons after reduced food intake (Ebling et al, 1990). Similarly, in hypogonadotropic lambs subjected to reduced diet, bolus of aspartate derivatives resulted in increased plasma LH concentrations (Ebling et al, 1990). Aspartate also stimulated LH secretion in gluco-deprived lambs (Bucholtz et al., 1996).

Similarly, an interaction of kisspeptin with GABA has been observed in pre-pubertal monkeys, bicuculline, GABA antagonist administration, aroused kisspeptin release (Kurian et al., 2012). It has been reported that in male Wistar rats short term fasting did not affect the *GAD67* mRNA expression in the brain tissue (Schwartz et al., 1993), whereas in another study long term fasting increased hypothalamic *GAD67* mRNA content of male rats (Leonhardt et al., 1999). It has been well known that *Kiss1* and *Kiss1r* expression decreases in the monkey hypothalamus after 48 h fasting (Wahab et al., 2011) but the actual mechanisms regulating this suppressed kisspeptin signaling during short term fasting are unknown. We designed the present study to examine the effects of excitatory (glutamate) and inhibitory (GABA) neurotransmitters on kisspeptin neurons during short term fasting. We hypothesized that: suppression of kisspeptin signaling pathway during short term fasting is due to a

decrease in excitatory and an increase in inhibitory inputs to kisspeptin neurons. In this study, we have selected *NR1* (an obligatory subunit of NMDA receptor) and *GAD67* (GABA synthesizing enzyme) genes to measure the changes in glutamate action and GABA synthesis during normally fed and 48 h fasting states. Therefore, the present study was designed to measure the correlative changes in the expression of *Kiss1, Kiss1r, NR1* and *GAD67* genes and changes in the kisspeptin neurons and NR1 expression in the hypothalamus of adult male rhesus macaque during fed and 48 h fasted states.

MATERIALS AND METHODS

Animals

Four adult male rhesus monkeys (*Macaca mulatta*) were used in this study. Body weight of these animals was 6.3-9.4 kg. These animals were placed in separate cages, in semi-controlled environment in the Primate Facility of the Department of Animal Sciences. Fresh fruits (0900-0930 h), boiled eggs (1100 h) and bread (1300-1330 h) were provided daily. Water was provided *ad libitum*. All the experimental procedures and protocols were permitted by the Care and Use of Animals Committee of the Department of Animal Sciences.

Tissue Collection and Blood Sampling

Hypothalamic blocks and blood samples were collected from four adult male rhesus monkeys. Two animals were normally fed whereas two animals were 48 h fasted but provided with water *ad libitum* at the time of euthanasia. Ketamine hydrochloride (Ketamax, Trittau, Germany; 10-20 mg/kg BW, im) was administered to sedate animals. After opening of skull, sodium thiopental (30 mg/kg BW; Abbott Laboratories, Karachi, Pakistan) was also injected intravenously. The brains were removed from the cranium immediately. Hypothalamic blocks including POA and MBH were dissected out from the brain by following the dissection procedure as described for rhesus monkey previously (Shibata et al., 2007). Briefly, taking median eminence as a central point, mammillary bodies (posteriorly) and anterior commissure (anteriorly) as landmarks, one coronal cut was made rostral to the anterior commissure and the other through the mammillary bodies. Then, two parasagittal cuts were made at about 5 mm on both sides of midline. At the caudal boundary of optic chiasm, a last cut dissected out square hypothalamic block. Further hemi-

hypothalamic blocks including MBH and POA were made by a cut along the medial line. Hemi-hypothalamic blocks were fixed in a fixative for immunocytochemistry and other hemi-hypothalamic blocks were quickly immersed in liquid nitrogen. Single blood samples were also collected from total of five animals for testosterone and glucose measurement, both during fed and fasting conditions. Blood sampling and tissue collection were done at day time (0010-0012 h) to make sure that all the experimental conditions were uniform. These experiments were carried out during the 3^{rd}-4^{th} week of February 2014. At this time the animals had ended their active breeding season.

Immunocytochemistry

Hemi-hypothalamic blocks of all animals were fixed in 4% paraformaldehyde solution in 0.1M PBS, (pH 7.4) for 14-18 h at 4˚C. Then hypothalamic blocks were transferred to 20% sucrose solution for 3 days at 4˚C and then to 30% sucrose solution at 4˚C until they sank. Consecutive 25 µm thick sections all over the whole hypothalamic block were cut on a cryostate (Bright OTF 5000; Bright Instrument Company, Huntingdon, UK) and stored in a cryopreservative solution (1% polyvinylepyrrolidone, 30% ethylene glycol and 30% sucrose in PBS) at -20˚C until used for immunohistochemistry.

Four free floating sections from each animal were processed for immunofluorescence to view the kisspeptin and NR1 immunoreactivity in the monkey hypothalami. One section from each animal was also processed as a primary antibody omitted control. Subsequent to 50 mM PBS washes (pH 7.3; 8×15 min) at room temperature, sections were incubated in incubation solution containing 10% normal donkey serum and 10% normal rabbit serum to block non-specific binding, on a shaker for one hour at room temperature. Then, sections were incubated in a cocktail of primary antibodies (GQ2;

1:120,000; kindly donated by Dr. Stephen Bloom of Imperial College London, UK and NMDAR1; 1:250; purchased from Chemicon, Temecula, California, USA) diluted in incubation solution containing 10% normal rabbit and 10% normal donkey serum for 48 h at 4˚C on a shaker. It was followed by 4 washes in 50mM PBS, each for 10 min at room temperature. To perceive immunofluorescence, sections were incubated in secondary antibodies (CF488 rabbit anti-sheep and Cy3-conjugated donkey anti-mouse) to identify kisspeptin and NR1 immunoreactivity, respectively, for 2 h in the dark at room temperature. Then the sections were washed in PBS (4 x 5 min) and mounted on super frosted slides (Marienfeld GmbH, Lauda-Königshofen, Germany) and coverslips were placed using Immunomount (Thermo Electron Corporation, Pittsburgh, USA) as a mounting medium. After mounting, slides were left overnight at 4˚C for drying in dark and stored at 4˚C until microscopy. To check the specificity of GQ2 and NR1, primary antibodies were omitted from the control sections.

Microscopy

Kisspeptin and NR1 immunoreactivity was viewed using Olympus fluorescent microscope (Olympus BX51, Tokyo, Japan) and photographs were taken using a digital camera attached to the microscope. From each animal, four random sections containing MBH were selected for the analysis. Three sections were treated with primary antibody and one was the primary antibody omitted control. Kisspeptin cells were stained green and NR1 expressing elements were appeared as red. Whole MBH area was scanned in three random sections from each animal. Total number of kisspeptin cells in each section was counted and mean ± SEM number of kisspeptin cells was calculated for each animal. Kisspeptin cells expressing NR1 were determined based on the analysis of merge images. Such double-labeled cells

appeared to express yellow immunoreactivity. As the three random sections were selected from each animal therefore the number was not constant.

SYBR Green qRT-PCR

I Isolation of RNA and cDNA Synthesis

Whole RNA was removed from hemi-hypothalamic block of normally fed and 48 h fasted adult male rhesus monkeys with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) by using provider's protocol. RNA quantity was measured by using a Thermo Scientific Nanodrop 1000 spectrophotometer (Wilmington, DE, USA). cDNA was synthesized from RNA by using First Strand cDNA Synthesis kit (Fermentas, Osaka, Japan), according to the protocol provided by the supplier with the random hexamer primers. Briefly, the process involved initial incubation for 10 min at 25˚C, then further incubation at 42˚C for 60 min followed by termination for 10 min at 70˚C. The cDNA samples were placed at -20˚C until further analysis.

II Relative Quantitative Real-time PCR

Expression of *Kiss1*, *Kiss1r*, *NR1* and *GAD67* genes were evaluated by the use of real-time polymerase chain reaction. The reactions were done by using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fischer Scientific, Osaka, Japan). Each reaction of 25 µl included 12.5 µl of SYBR Green, 0.5 µl of each primer and 10 µl of PCR water. The primer sequences were synthesized by Fermentas Thermo Fischer Scientific (Osaka, Japan). Sequences of all used primers are as follows *Kiss1r* F 5-AACTCGCTGGTCATCTACGTCAT-3, R 5-TCTGTGGCCGCCAGGTT-3 (XM 001117198.1; product size 86); *Kiss1* F 5-AGAAAAGGTGGCCTCTGTGGA-3, R 5-AGGCTCTGCTCCCACGG-3 (XM 001098284.2; product size 87); *GAD67* F 5-GAGGACCCCGGACAGTAGAGGC-3, R 5-GATCTTGAGCCCCAGTTTTCTG-3 (XM 001082995.2; product size 181); *NR1* (GRIN1) F 5

AGGAACCCCTCGGACAAGTT-3, R 5-CGAGTCCCAGATGAAGGCA -3 (XM 001117773.2; product size 183). *GAPDH* F 5-AAGTACGATGACATCAAGAAG-3, R 5-GAAGAGTGGGTGTCGCTGTTG-3 (NM 001195426.1; product size 119 bp). Thermocycler conditions were first a hot start at 95˚C for 10 min then 40 cycles each at 95˚C for 15 sec followed by 60˚C for 30 sec and 72˚C for 30 sec. All reactions were run in duplicate and cycle threshold (Ct) was calculated by using software (Rotor gene 3000 Real time PCR machine, Corbett Research, Australia). Comparative expression was calculated by using the relative cycle threshold (Ct) method. Every sample was normalized to endogenous housekeeping gene *GAPDH* expression by using the 2 delta Ct method.

Blood Glucose and Plasma Testosterone Measurement

Plasma concentration of total testosterone was measured by using a human testosterone ELISA Kit (Amgenix International Inc., San Jose, CA, USA). Assay sensitivity was 0.05 ng/ml, and intra-assay coefficient of variation was less than 10%. Blood glucose was measured by placing a drop of blood on the strip of Oncall plus blood glucometer (ACON laboratories, San Diego, CA, USA).

Statistical Analyses

Data are presented as mean ± SEM. Mean values in fed and fasting conditions were compared by applying student-t test. Differences were measured to be statistically significant when $P<0.05$.

RESULTS

Hypothalamic tissues were taken from four adult male rhesus monkeys during fed and 48 h fasting states. Blood glucose levels during fed and 48 h fasting conditions are shown in the Figure 2.1. Mean plasma testosterone concentrations in fed and 48 h fasted animals are shown in Figure 2.2. Plasma testosterone and blood glucose levels were decreased in monkeys which were subjected to 48 h fasting as compared to those monkeys which were fed *ad libitum*. Relative quantity of *Kiss1*, *Kiss1r*, *NR1* and *GAD67* mRNA contents are shown in the Figures 2.3-2.6. *Kiss1*, *Kiss1r* and *NR1* mRNA expression levels were significantly ($p<0.05$) reduced after 48 h of fasting in the hypothalamus of adult male macaques. In contrast, *GAD67* mRNA expression levels were not changed between the two groups. Individual and mean numbers of single and double labeled kisspeptin neurons are shown in Tables 2.1 and 2.2. Dual label immunofluorescence of kisspeptin and NR1 in hypothalamus of adult male rhesus macaques during fed and 48 h fasted states are shown in Figures 2.7-2.8. In ARC region, kisspeptin neurons showed strong interaction with NR1 expressing elements in monkeys fed *ad libitum,* evidenced by the appearance of yellow colour at the point of co-localization of kisspeptin (green) and NR1 (red). This interaction of kisspeptin neurons with NR1 subunit of NMDA receptor was significantly ($p<0.05$) reduced after short term fasting (Figure 2.9).

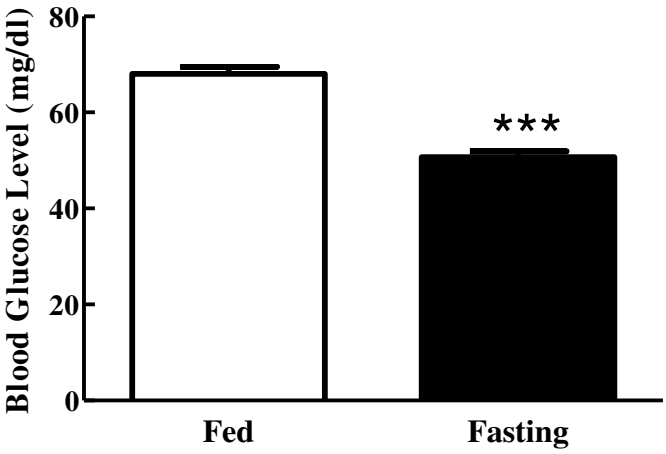

Figure 2.1. Mean (±SEM) blood glucose levels observed in fed and 48 h fasting adult male monkeys (n=3). Short term fasting significantly (***p<0.001) decreased blood glucose levels.

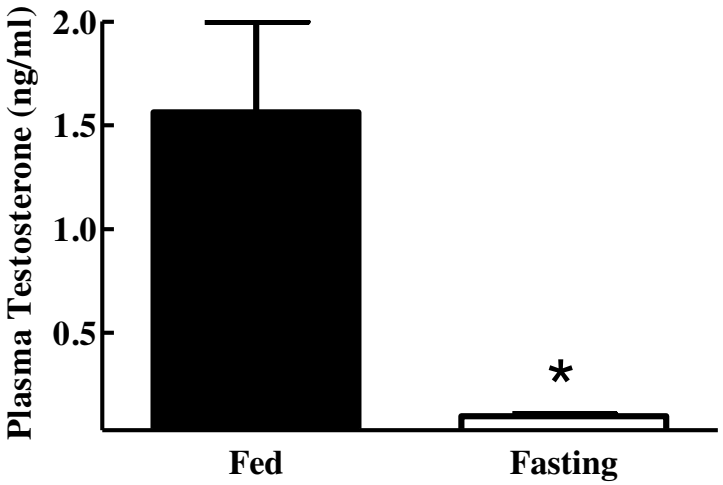

Figure 2.2. Mean (±SEM) plasma testosterone levels observed in fed (n=5) and fasting adult male rhesus monkeys (n=5). 48 h fasting significantly (*$p<0.05$) decreased plasma testosterone levels.

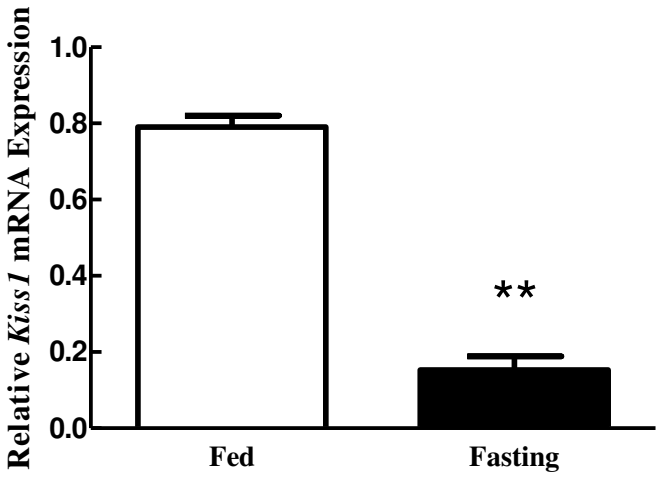

Figure 2.3. Mean (±SEM) relative hypothalamic *Kiss1* mRNA expression levels in adult male monkeys which were subjected to 48 h fasting (n=2) and normal feeding (n=2). *Kiss1* mRNA contents were significantly (**p<0.01) decreased in rhesus monkeys deprived of food for 48 h as compared to those monkeys which were fed *ad libitum. GAPDH* was used as a housekeeping gene to normalize the *Kiss1* mRNA contents.

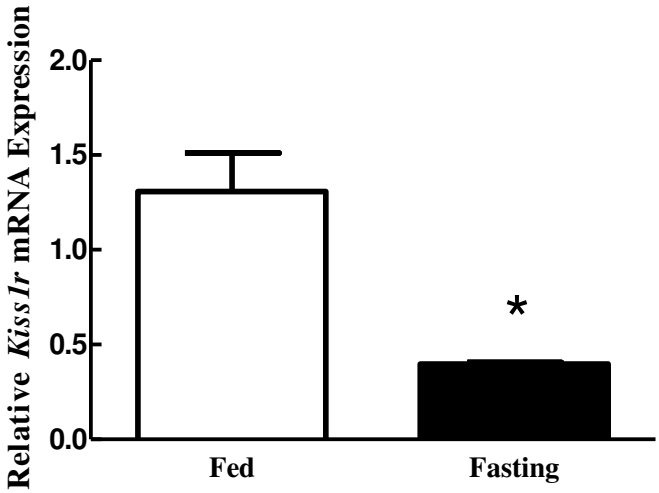

Figure 2.4. Mean (±SEM) relative hypothalamic *Kiss1r* mRNA expression levels in adult male monkeys which were subjected to 48 h fasting (n=2) and normal feeding (n=2). *Kiss1r* mRNA contents were significantly (*$p<0.05$) decreased in rhesus monkeys deprived of food for 48 h as compared to those monkeys which were fed *ad libitum. GAPDH* was used as a housekeeping gene to normalize the *Kiss1r* mRNA contents.

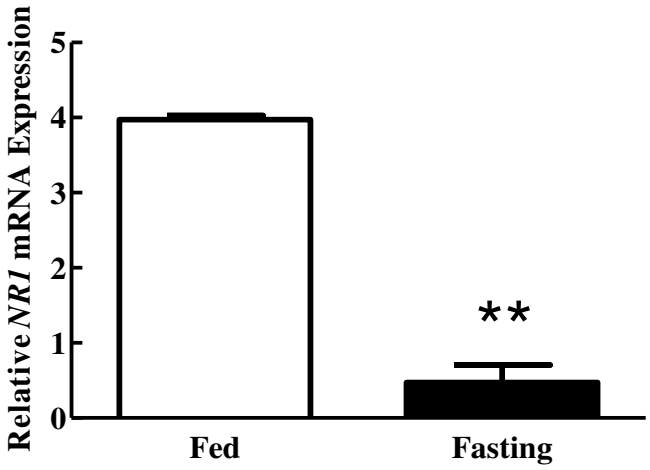

Figure 2.5. Mean (±SEM) relative hypothalamic *NR1* mRNA expression levels in adult male monkeys which were subjected to 48 h fasting (n=2) and normal feeding (n=2). *NR1* mRNA contents were significantly (**p<0.01) decreased in rhesus monkeys deprived of food for 48 h as compared to those monkeys which were fed *ad libitum*. *GAPDH* was used as a housekeeping gene to normalize the *NR1* mRNA contents.

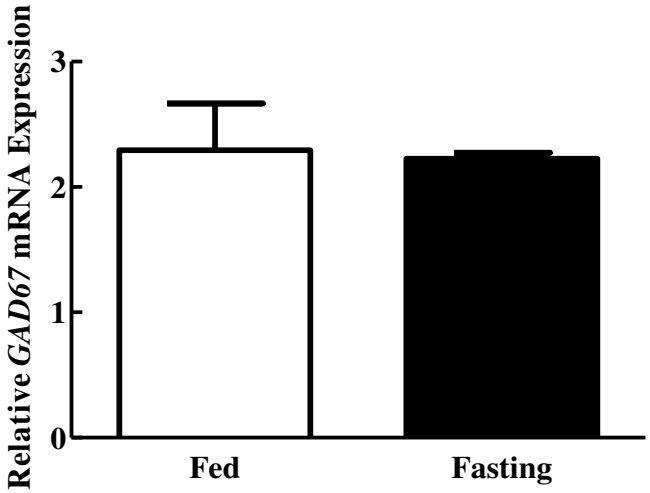

Figure 2.6. Mean (±SEM) relative hypothalamic *GAD67* mRNA expression levels in adult male rhesus monkeys which were subjected to short term fasting (n=2) and normal feeding (n=2). *GAD67* mRNA contents were similar between the two groups. *GAPDH* was used as a housekeeping gene to normalize the *GAD67* mRNA contents.

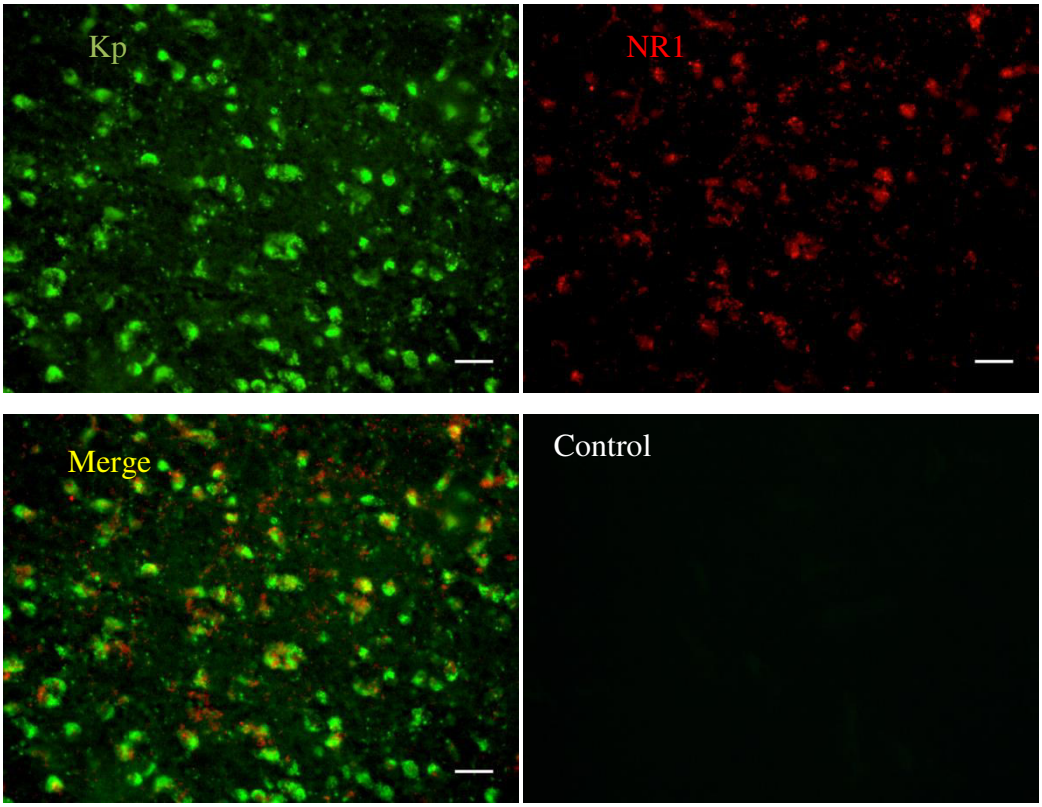

Figure 2.7. Fluorescent microscopic images showing kisspeptin immunoreactivity in green (upper left) and NR1 subunit of NMDA receptor in red (upper right) in the MBH of male rhesus macaque during fed condition. Merge image of kisspeptin and NR1 (lower left) showing co-localization (yellow colour) and primary antibody omitted control (lower right). Photographs were taken at 200x magnification.

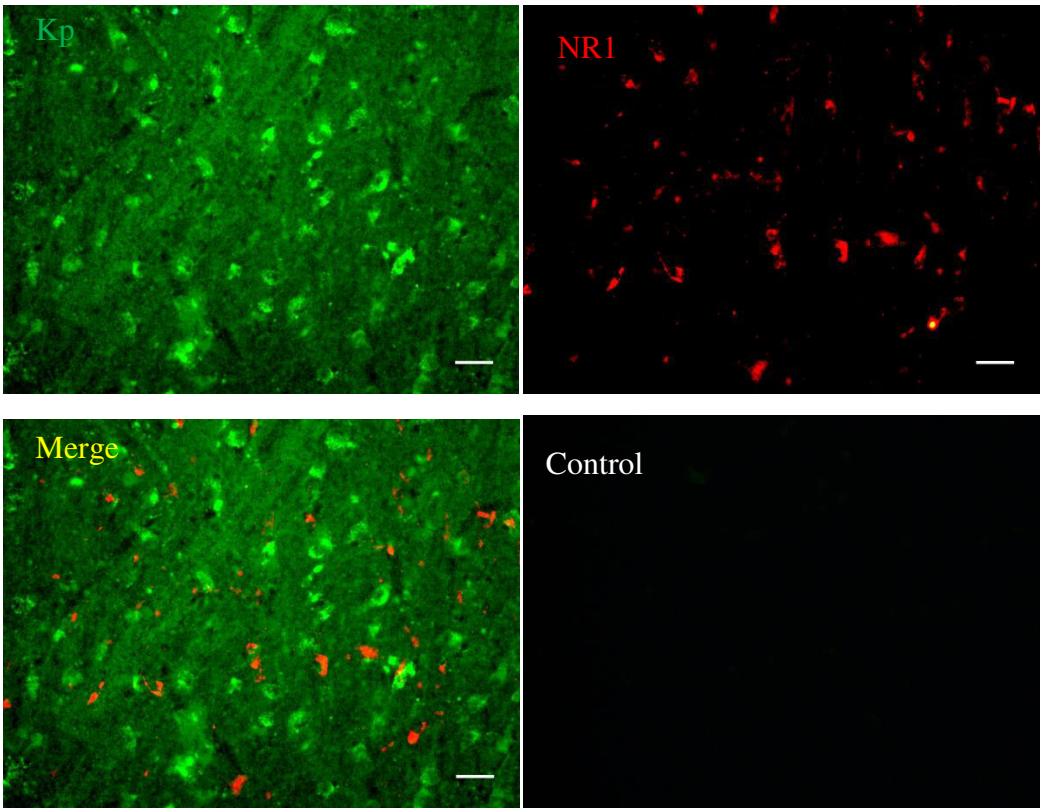

Figure 2.8. Fluorescent microscopic images showing kisspeptin immunoreactivity in green (upper left) and NR1 in red (upper right) in the MBH of adult male rhesus macaque after 48 h fasting at 200x magnification. Merge image of kisspeptin and NR1 (lower left) showing co-localization (yellow colour) and primary antibody omitted control (lower right).

Table 2.1. Comparison of individual and mean number of single (kp) and double labeled (dbl) kisspeptin neurons expressing NR1 in the MBH of three sections in adult male rhesus macaque (n=2) during fed state

Animal No.	Number of kp neurons during normally fed condition						Mean Kp	Mean dbl	% dbl
	Section 1		Section 2		Section 3				
	Kp	dbl	Kp	dbl	Kp	dbl			
M2	180	30	155	16	170	35	168.33	27.00	16.04
M7	160	23	159	21	140	33	153.00	25.67	16.76
Mean							160.67	26.33	16.41
±SEM									16.70±0.07

Table 2.2. Comparison of individual and mean number of single (kp) and double labeled (dbl) kisspeptin neurons expressing NR1 in MBH of three sections of adult male rhesus macaque (n=2) subjected to 48 h fasting

Animal #	Number of kp neurons after 48h fasting						Mean Kp	Mean dbl	% dbl
	Section 1		Section 2		Section 3				
	Kp	dbl	Kp	dbl	Kp	dbl			
M8	118	13	112	10	70	14	100.00	12.33	12.33
M9	96	12	83	16	100	05	93.00	11.00	11.82
Mean							96.50	11.67	12.08
±SEM									12.08±0.25

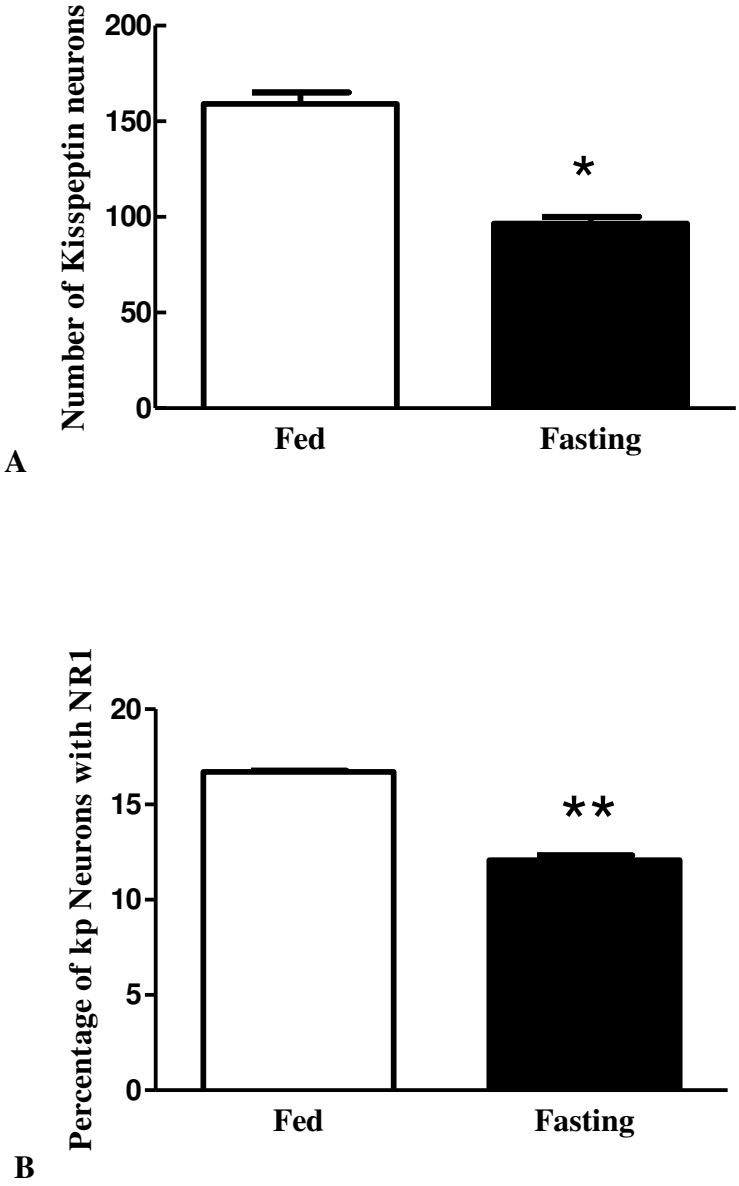

Figure 2.9. Comparison of the number of kisspeptin neurons (**A**) and percentage of kisspeptin neurons expressing NR1 (**B**) in MBH of adult male rhesus monkeys during fed (n=2) and fasting (n=2) conditions. Number of kisspeptin neurons (*$p<0.05$) and percentage of kisspeptin neurons expressing NR1 (**$p<0.01$) significantly reduced under short term fasting condition.

DISCUSSION

In recent times, a large number of studies have shown that decreased hypothalamic *Kiss1* mRNA expression in rodents is associated with attenuated HPG axis during short term fasting (Castellano et al., 2009). Exogenous administration of kisspeptin during short term fasting restores HPG axis by stimulating the secretion of reproductive hormones (Castellano et al., 2009). Recently, Wahab et al. (2008) have shown that short term fasting induced suppression of HPG axis is reversed by exogenous kisspeptin administration in adult male monkeys. The results of this study show that 48 h fasting in intact adult male rhesus monkey induces the suppression of HPG axis, which is revealed by the decrease in plasma testosterone concentrations after 48 h fasting. Out of various metabolic fuels, glucose plays an imperative role in the regulation of reproductive axis (Ohkura et al., 2000; Oltmanns et al., 2001; Lado-Abeal et al., 2002; Ohkura et al., 2004). In this study, blood glucose levels were significantly decreased after short term fasting in line with the previous studies (Heitmann et al., 1986; Beer et al., 1989; Fairhal et al., 1990; Barb et al., 2001). It has been hypothesized that kisspeptin is a common conduit, which relays metabolic status information to the brain regions which control HPG axis (Castellano et al., 2009). *Kiss1* and *Kiss1r* mRNA expression levels decrease during short term fasting, which may act in part to suppress HPG axis in rodents and primates (Smith et al., 2006; Luque et al., 2007; Castellano et al., 2009; Wahab et al., 2011). However, the actual mechanisms involved in the suppression of kisspeptin signaling pathway during short term fasting remain to be elucidated. Therefore, this study has focused on analyzing the interplay of kisspeptin with two afferent neurotransmitters, which have been shown to influence the kisspeptin neurons, during fed and fasting conditions.

Our results of immunofluorescence assays show that kisspeptin peptide expression in the hypothalamus of adult male monkey decreased after 48 h fasting. The percentage of kisspeptin cells expressing NR1 also decreased after 48 h fasting. We also quantified *Kiss1*, *Kiss1r, NR1* and *GAD67* mRNAs in the hypothalamus of adult male rhesus macaque subjected to 48 h fasting and fed *ad libitum*. Our results indicate that hypothalamic *Kiss1*, *Kiss1r* and *NR1* mRNA contents are significantly reduced in the 48 h fasted animals than those fed *ad libitum*. Whereas, hypothalamic *GAD67* mRNA expression levels were not altered between the two groups. This suggests that decreased kisspeptin signaling during short term fasting might be due to decrease in the NMDA dependent excitatory inputs to kisspeptin neurons. These findings are consistent with the previous observations that HPG axis is less responsive to NMDA administration after short term fasting (Shahab et al., 1997), and *GAD67* mRNA expression remains unchanged after short term fasting in male Wistar rats (Schwartz et al., 1993).

Kisspeptin signaling is strongly modulated by excitatory and inhibitory neurotransmitters. It is further supported by the observation of activation of ARC-KNDy neurons (Cheng et al., 2010) by NMDA and inhibition by GABA (Kurian et al., 2012; Gottsch et al., 2011; Qiu et al., 2011). Electrophysiological studies have shown that RP3V-Kiss1 neurons are also strongly activated by glutamate and inhibited by GABA (Ducret et al., 2010).

It is likely that reduced kisspeptin signaling and suppressed HPG axis during short term fasting is mediated by attenuated excitatory afferents to kisspeptin neurons. This speculation is supported by the observation that HPG axis becomes less responsive to exogenous NMDA administration during fasting (Shahab et al., 1997).

This decrease in the hypothalamic *NR1* mRNA expression levels may contribute to the reduction of kisspeptin signaling during short term fasting in non-human primates. On the other hand, the *GAD67* mRNA expression levels were similar between fed and fasted groups in the current study. Similar to our findings the *GAD67* mRNA expression levels were not influenced by metabolic state in male Wistar rats (Schwartz et al., 1993). In contrast to our findings, long term fasting resulted in enhancement of *GAD67* mRNA levels in male rats (Leonhardt et al., 1999). This might be due to the long-term deprivation of food intake. Our results demonstrate that GABA is not involved in the short term fasting induced suppression of kisspeptin signaling.

In summary, the reduction of kisspeptin expression during short term fasting appears to be associated with decreased glutamate action as indicated by a decrease in *NR1* gene expression and a decrease in NR1 expression on kisspeptin neurons in adult male monkey hypothalamus. Furthermore, considering the fact of unchanged *GAD67* mRNA expression levels in fasting, it is likely to conclude that fasting induced suppression of kisspeptin signaling is not mediated through GABAergic actions. Our results suggest that GABA synthesis is not affected by short term fasting and hence, it is not involved in fasting induced suppression of kisspeptin signaling. We also suggest that NMDA acts through NMDA receptor to control kisspeptin signaling in rhesus monkey during fed and fasting conditions.

Chapter 3

Studies on the neurotransmitter regulation of kisspeptin neurons

Differential expression of estrogen receptor α (ERα) and androgen receptors (AR) on kisspeptin neurons of rostral periventricular area of third ventrical (RP3V) in adult male and female mice

ABSTRACT

Background: Kisspeptin is a neuropeptide encoded by the *Kiss1* gene and acts through the activation of GPR54. The most important function of kisspeptin neurons is to stimulate GnRH secretion which further regulates the reproduction. Although a lot of studies have been done regarding the kisspeptin regulation of HPG axis but very scarce information is available about the regulation of kisspeptin itself.

Objective: Steroids regulate *Kiss1* expression in male and female mice. Specifically, whether testosterone or estradiol are involved in the steroid regulation of RP3V kisspeptin neurons is unknown. In addition, whether there is any difference in the percentage of kisspeptin neurons (residing in RP3V region) expressing ERα or AR in male and female mice, remains to be elucidated. Therefore, this study was designed to observe the expression of estradiol or testosterone receptors on kisspeptin neurons in both adult male and female mice.

Materials and Methods: Whole brains were removed from transcardialy perfused ten adult male and ten adult female C57BL/6J mice. Dual label chromogen immunocytochemistry was performed by using specific primary antibodies directed against kisspeptin and ERα/AR in RP3V region of male and female mice. Kisspeptin immunoreactivity was revealed by treating the brain sections with DAB (brown cytoplasmic staining) and ERα/AR immunoreactivity was revealed by treating the brain sections with NiDAB (black nucleus staining).

Results: The results of these experiments demonstrated that RP3V kisspeptin neurons expressed ERα and AR in both male and female mice. Overall 75% and 11.9% of kisspeptin neurons expressed AR in RP3V of adult male and female mice,

respectively. Percentage of RP3V kisspeptin neurons expressing AR was significantly greater ($p<0.05$) in male mice as compared to female mice. About 65% and 37% of kisspeptin neurons expressed ERα in RP3V of adult male and female mice, respectively. However, this difference in percentage of RP3V kisspeptin neurons between male and female mice was statistically non-significant ($p>0.05$). Percentage of ERα expressing kisspeptin neurons was significantly ($p<0.05$) higher than the percentage of kisspeptin neurons expressing AR in female mice. However, the percentage of kisspeptin neurons expressing ERα and AR was statistically similar in male mouse. On the basis of the current finding that 75% of kisspeptin cells expressed AR and 65% of kisspeptin cells expressed ERα, we assume that there might be one population of RP3V kisspeptin neurons in male mice, which express both AR and ERα.

Conclusion: Our findings extend our knowledge about the steroid regulation of kisspeptin neurons in rodents, indicating the possibility that both estradiol and testosterone are involved in steroid regulation of this particular population of kisspeptin neurons in male and female rodents.

INTRODUCTION

Secretion of GnRH during late postnatal life is critical for fertility and initiation of puberty (Terasawa and Fernandez, 2001; Plant and Witchel, 2006). The secretion of GnRH neurons increases at the time of puberty due to decrease in inhibitory inputs and increase in excitatory inputs to GnRH neurons (Ojeda and skinner, 2006; Ojeda et al., 2006). Glutamate and GABA are important excitatory and inhibitory inputs (Ojeda and Skinner, 2006; Ojeda et al., 2006).

There are many neurotransmitters and neuropeptides, which stimulate the secretion of GnRH (Brann, 1995; Ojeda and Skinner, 2006; Plant and Witchel, 2006). The most important neuropeptide, which is proved to be important for the stimulation of GnRH neurons is kisspeptin. It is encoded by *Kiss1* gene and acts through the activation of previously orphan G-protein coupled receptor. Initially, it was discovered as a metastasis suppressor sequence (Lee et al., 1996). But later on in 2003, its role in reproduction was revealed in IHH patients having *KISS1R* mutations (de Roux et al., 2003; Seminara et al., 2003). Similarly, targeted deletions of *Kiss1r* in mice result in IHH (Funes et al., 2003) and activating mutations of *KISS1R* result in precocious puberty in girls (Teles et al., 2008). These findings propose that kisspeptin signaling is important for fertility and reproductive functions (reviewed in d'Anglemont de Tassigny et al., 2007; Oakley et al., 2009).

Kisspeptin neurons are found to be located in two distinct regions of mouse brain: one neuronal population is present in RP3V region and the other in the ARC (Gottsch et al., 2004; Clarkson and Herbison, 2006; Clarkson et al., 2009b). It appears that RP3V kisspeptin neuronal population is important for stimulation of GnRH neurons at the time of puberty, which is revealed by the increase in kisspeptin neuronal number

throughout pubertal development and increase in kisspeptin fiber appositions with GnRH neurons in a rostral POA (Clarkson and Herbison, 2006). In addition, it has been found that kisspeptin neurons stimulate GnRH neurons directly (Liu et al., 2011). These observations propose that kisspeptin is necessary for the stimulation of GnRH neurons. It has been found recently that increase in RP3V kisspeptin expression at the time of puberty is dependent upon estradiol in female mice (Bakker et al., 2010; Clarkson et al., 2009b).

Ovariectomy, results in the reduction of kisspeptin neurons in RP3V whereas estradiol treatment restores kisspeptin neuronal number in RP3V region of ovariectomised mice (Clarkson et al., 2009b). However, ARC kisspeptin neurons are negatively regulated by estradiol as shown by ovareictomised female and castrated male mice (Smith et al., 2005b), rhesus monkey (Rometo et al., 2007; Shibata et al., 2007) and sheep (Pompolo et al., 2006), which contain high levels of the *Kiss1* mRNA in the neurons as compared to intact control groups. In addition, ERα knockout mutation stops pubertal development of RP3V kisspeptin neurons (Mayer et al., 2010). These observations show that steroids positively regulate RP3V kisspeptin neurons in female mice. It has been well established that kisspeptin neurons are sexually dimorphic having more number in females as compared to males but the pattern of the development is similar in both male and female mice (Clarkson and Herbison et al., 2006). Similarly, gonadal steroids stimulate RP3V kisspeptin neurons during pubertal development in male mice (Clarkson et al., 2012).

It has been well established that kisspeptin is the powerful regulator of GnRH neurons and steroids regulate kisspeptin expression in male and female mice. Specifically whether testosterone or estradiol is involved in the steroid regulation of kisspeptin neurons is unknown. Therefore, this study was designed to examine the expression of

estradiol or testosterone receptors on kisspeptin neurons in adult male and female mice. To extend our knowledge about the role of steroids in regulation of kisspeptin neurons in RP3V brain region of male and female mice, we have undertaken dual label immunocytochemical studies for kisspeptin and Erα or AR in adult male and female mice.

MATERIALS AND METHODS

Animals

In this study ten adult male and ten adult female (n=10) C57BL/6J mice were used. These animals were kept under 12-h light and 12-h dark cycle (lights were on at 6:00 or 6:30 AM). All mice were provided with water and food *ad libitum*. The experiment was carried out at the Laboratory of Dr. Allan Herbison, Department of Physiology, School of Medical Sciences, University of Otago, Dunedin, New Zealand during the period from July 2010 to December 2010, when the author was availing six month training under IRSIP program of the HEC. All of the experimental protocols were permitted by the Animal Welfare and Ethics Committee of the University of Otago, Dunedin, New Zealand.

Tissue Collection

All animals were trans-cardialy perfused with 4% paraformaldehyde solution. Then brains were taken out from the skull and fixed in 4% paraformaldehyde solution for 1h. After fixation, brains were placed in 30% sucrose solution for the whole night at 4˚C. Three sets of 30µm thick sections were cut on a sliding freezing microtome (Leica SM2400*;* Leica, Nussloch, Germany) from the forebrain to the end of the brain stem and placed in TBS buffer at 4˚C until processed for immunocytochemistry.

Co-localization of Kisspeptin Neurons and Androgen Receptors in RP3V of Male and Female mice

One set of free floating sections was processed in 12 well culture plates for kisspeptin and AR dual label chromogen immunocytochemistry. Initially, sections were placed in 3% hydrogen peroxide (H_2O_2) solution containing 11.4 ml of TBS and 8 ml of

methanol to reduce endogenous peroxidase activity for 10 min at room temperature. Then the sections were given three TBS washes each for 10 min. Sections were then placed in rabbit polyclonal primary antibody specific for androgen receptors (kindly provided by Dr. Gail Prins, University of Illinois, Chicago, IL, USA; 1:500) for 48 h at 4˚C. This antibody has been described previously (Prins et al., 1991). The primary antibody was diluted in incubation solution and 2% normal goat serum. After three washes in TBS each for 10 min, sections were incubated in secondary antibody, biotinylated goat anti-rabbit (1:200; Vector Laboratories, Burlingame, CA, USA), for 90 min at room temperature. Following three TBS washes, sections were placed in Vector Elite avidin-peroxidase (1:100; Vector Laboratories) solution for 90 min at room temperature. Subsequent to three washes in TBS, sections were treated with glucose-oxidase and Ni (12.5 mg/ml; BDH Laboratory Supplies) DAB (Sigma Chemical Company; St. Louis, MO, USA) that stained nucleus of the labeled cell (black colour). For double labeling, sections were again treated with 3% hydrogen peroxide to block endogenous peroxidases. After three washes in TBS each for 10 min, sections were incubated in a polyclonal rabbit anti-kisspeptin antibody (1:10,000; donated by Dr. Alain Caraty, INRA) diluted in incubation solution and 2% normal goat serum for 48 h at 4°C. Characteristics of this primary antibody have been previously described (Desroziers et al., 2010). Subsequent to three washes in TBS, sections were treated with peroxidase-labeled goat anti-rabbit secondary antibody (1:400; Vector Laboratories) solution for 4 h at room temperature. After washing in TBS, sections were treated with glucose-oxidase and DAB (Sigma), which stained cytoplasm (brown colour). Sections were then washed in TBS and mounted on gelatin coated glass slides and air dried overnight. Then, sections were passed through ascending grades of alcohol (50, 70, 95, 100, 100%) followed by two xylene washes.

Sections were then coverslipped by using DPX (BDH Laboratory Supplies) mounting media. Four primary antibody omitted control sections were also processed.

Co-localization of Kisspeptin Neurons and Estrogen Receptors α in RP3V Region of Male and Female Mice

Another set of free-floating sections was processed for kisspeptin and ERα dual label chromogen labeling. Initially sections were placed in 3% hydrogen peroxide (H_2O_2) solution containing 11.4ml of TBS and 8ml of methanol to reduce endogenous peroxidase activity for 10min. Sections were then given three TBS washes each for 10 min and treated with rabbit polyclonal primary antibody for ERα (1:10,000; Millipore, Billerica, MA, USA) for 48h at 4˚C. The primary antibody was diluted in incubation solution and 2% normal goat serum. After three washes in TBS each for 10min, sections were treated with biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) for 90 min at room temperature. Following three TBS washes, sections were placed in Vector Elite avidin–peroxidase (1:100; Vector Laboratories) solution for 90 min at room temperature. Subsequent to three washes in TBS, sections were treated with glucose-oxidase and Ni (12.5 mg/ml; BDH Laboratory Supplies) DAB (Sigma) that stained nucleus of the labeled cell (black colour). For double labeling, endogenous peroxidases were blocked by treating the sections with 3% hydrogen peroxide. After three TBS washes, sections were then treated with polyclonal rabbit anti-kisspeptin antibody (1:10,000; donated by Alain Caraty, INRA) solution, diluted in incubation solution and 2% goat serum for 48 h at 4°C. Subsequent to three washes, sections were treated with peroxidase-labeled goat anti-rabbit immunoglobulins (1:400; Vector Laboratories) for 4 h at room temperature. After washing in TBS, sections were treated with glucose-oxidase and

DAB (Sigma), which stained cytoplasm (brown colour). Sections were then washed with TBS and mounted on gelatin coated glass slides and air dried overnight. Then sections were passed through ascending grades of alcohol (50, 70, 95, 100, 100%) followed by two xylene washes. Sections were then coverslipped by using DPX (BDH Laboratory Supplies) mounting media. In the present experiments, antibody omitted sections had no immunoreactivity.

Microscopy

Double-labeled immunocytochemistry was examined under Olympus BX51 (Olympus, Tokyo, Japan) by using ordinary light. Kisspeptin neurons were stained as brown cytoplasm while ERα and AR were stained as black nuclei. Kisspeptin neurons were considered single labeled when only brown cytoplasm was visible whereas double-labeled kisspeptin neurons were stained as brown cytoplasm with black nucleus. Kisspeptin immunoreactivity was observed in the pre-optic periventricular nucleus (PVpo) and periventricular continuum of AVPV (Clarkson and Herbison, 2006) collectively called RP3V (Herbison, 2008). AVPV and PVpo anatomical description in C57BL/6J mice has been published recently (Herbison, 2008). In the present study, by using this map we have examined kisspeptin neurons in three levels of RP3V (AVPV, rostral PVpo and caudal PVpo). Total of six sections were selected from each animal. Two sections from each level of RP3V were examined for single and double-labeled quantification. Kisspeptin cells in each area of RP3V were counted and then added to find the mean number of kisspeptin cell in three regions of RP3V. Then the mean number of kisspeptin cells in three regions was combined to find the mean number of kisspeptin cells per section of RP3V. Mean data of each mouse were added to find mean ± SEM of the experimental group. Similarly,

percentage of double-labeled kisspeptin neurons was determined for each mouse and grouped to find mean ± SEM values for the experiment.

Statistical Analyses

Data are presented as mean ± SEM. Mean values of the percentage of ERα and AR expressing kisspeptin neurons in RP3V of male and female mice were compared by applying student-t test. Differences were measured to be statistically significant when $P<0.05$.

RESULTS

Co-localization of Kisspeptin and ERα in RP3V Region of Adult Male Mice

Kisspeptin immunoreactivity has been observed in AVPV, rPVpo and cPVpo regions as has been reported previously (Clarkson and Herbison, 2006a). Our results show that ERα has been expressed in many regions of hypothalamus such as organum vasculosum of lamina terminalis, medial POA, RP3V, BnST and anterior hypothalamus. Double-labeled chromogen immunocytochemistry showed the presence of many kisspeptin cells in RP3V of male mice. Kisspeptin neurons stained as brown cytoplasm while ERα immunoreactivity stained as black nucleus (Figure 3.1). Individual and mean number of single and double-labeled kisspeptin neurons expressing ERα in RP3V in the male mouse brain has been shown in Table 3.1. Percentage of double-labeled kisspeptin neurons with ERα was 65.3 ± 13.4%. Mean ± SEM number of kisspeptin neurons detected per section of RP3V was 1.4 ± 0.5.

Co-expression of Kisspeptin and AR in RP3V Region of Adult Male Mice

AR expression was observed in RP3V, anterior hypothalamus, medial POA and BnST. Kisspeptin neurons were stained brown whereas androgen receptors were stained black (Figure 3.2). Primary antibody omission resulted in the complete absence of staining. Individual and mean number of single and double-labeled kisspeptin neurons has been shown in Table 3.2. Percentage of double-labeled kisspeptin neurons in RP3V was 75.0 ± 21.7%. Mean number of kisspeptin cells per section of RP3V was 0.4 ± 0.1.

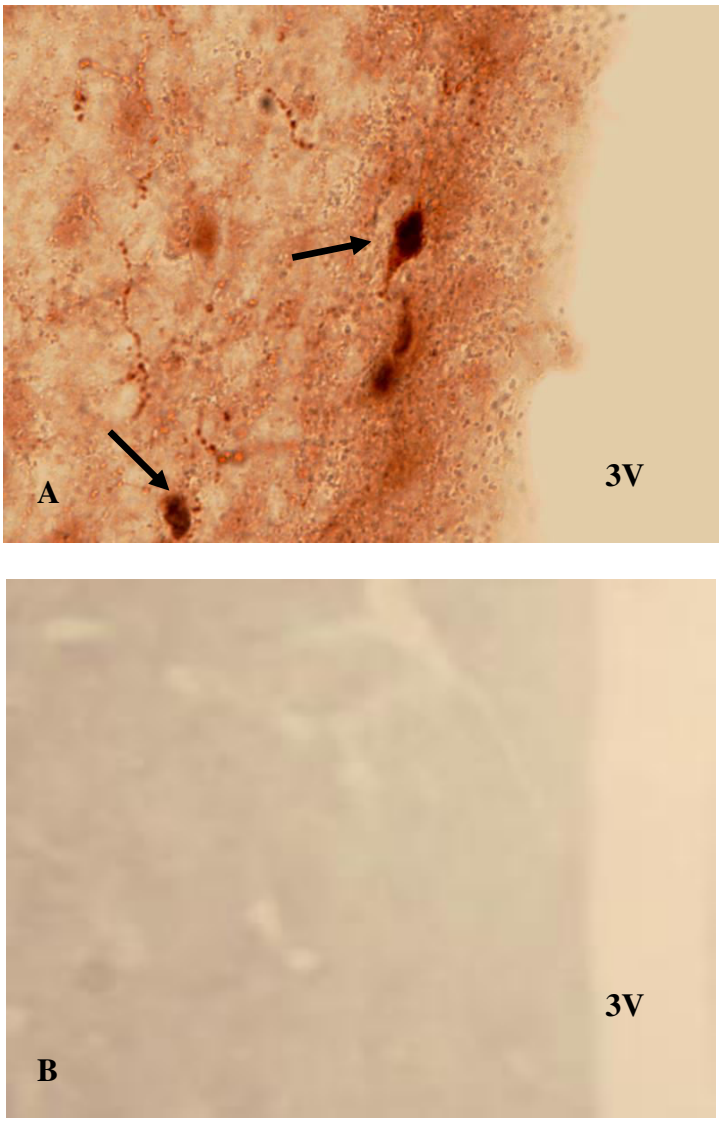

Figure 3.1. Representative photographs showing double chromogen labeling for kisspeptin neurons (brown) and ERα (black) in RP3V region of male mice at 600x (**A**) magnification and control (**B**). "3V" denotes the third ventricle. Kisspeptin immunoreactivity was performed by treating with DAB and ERα immunocytochemistry was performed by treating with NiDAB. Arrows indicate dual-labeled kisspeptin cells with ERα.

Table 3.1. Individual and mean number of single (kp) and double labeled (dbl) kisspeptin neurons expressing ERα observed in two sections from three regions of RP3V in the male mouse brain

Animal No.	Section1		Section2		mean kp/ section	mean dbl/ section	%dbl
	kp	dbl	kp	dbl			
M1							
AVPV	1	1	1	0	1.0	0.5	
rPVpo	3	3	0	0	1.5	1.5	
cPVpo	5	4	7	3	6.0	3.5	
Mean					2.83	1.83	64.70
M2							
AVPV	0	0	0	0	0.0	0.0	
rPVpo	1	1	0	0	0.5	0.5	
cPVpo	2	2	7	4	4.5	3.0	
Mean					1.67	1.67	70.00
M3							
AVPV	1	1	0	0	0.5	0.5	
rPVpo	2	0	2	1	2.0	0.5	
cPVpo	3	0	0	0	1.5	0.0	
Mean					1.33	0.33	25.00
M4							
AVPV	0	0	0	0	0.0	0.0	
rPVpo	1	0	0	0	0.5	0.0	
cPVpo	2	2	0	0	1.0	1.0	
Mean					0.50	0.33	66.67
M5							
AVPV	0	0	0	0	0.0	0.0	
rPVpo	0	0	0	0	0.0	0.0	
cPVpo	1	1	3	3	2.0	2.0	
Mean					0.67	0.67	100.00

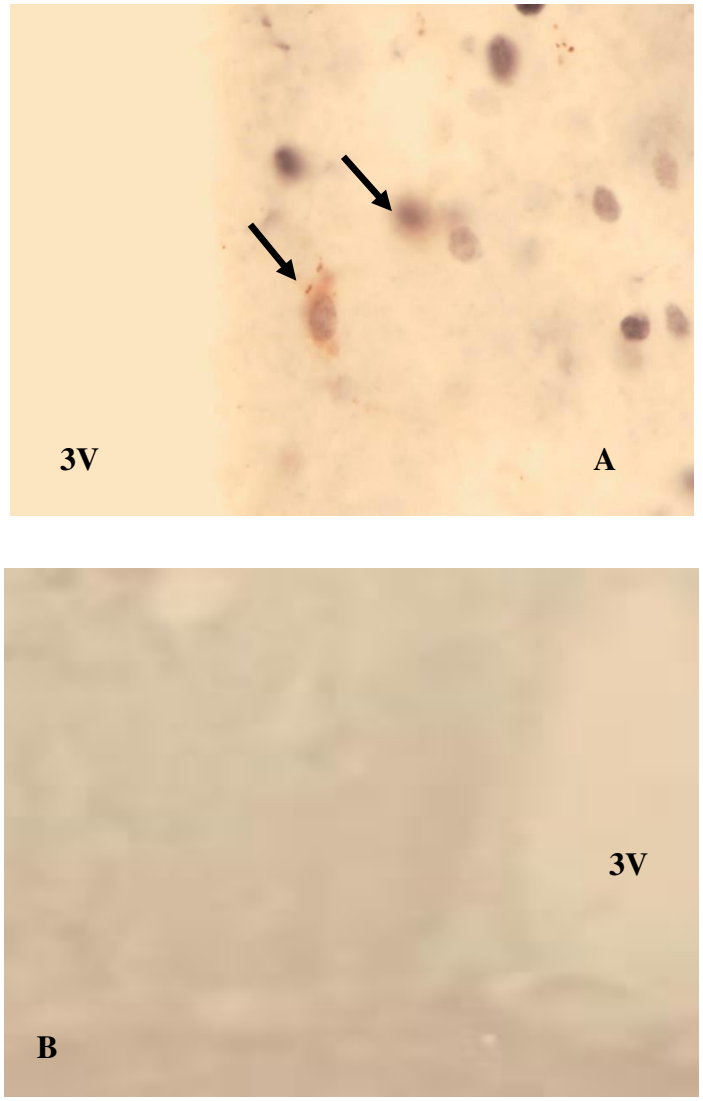

Figure 3.2. Representative photographs showing double chromogen labeling for kisspeptin neurons (brown) and AR (black) in RP3V region of male mice at 600x magnification (**A**) and control (**B**). "3V" denotes the third ventricle. Kisspeptin immunoreactivity was performed by treating with DAB and AR immunocytochemistry was performed by treating with NiDAB. Arrows indicate dual-labeled kisspeptin cells with AR.

Table 3.2. Individual and mean number of single (kp) and double-labeled (dbl) kisspeptin neurons expressing AR observed in two sections from three regions of RP3V in male mouse brain

Animal No.	Section1		Section2		mean kp/ section	mean dbl/ section	%dbl
	kp	dbl	kp	dbl			
M1							
AVPV	1	1	0	0	0.5	0.5	
rPVpo	0	0	0	0	0.0	0.0	
cPVpo	1	1	0	0	0.5	0.5	
Mean					0.33	0.33	100.00
M2							
AVPV	1	1	0	0	0.5	0.5	
rPVpo	2	1	0	0	1.0	0.5	
cPVpo	1	1	0	0	0.5	0.5	
Mean					0.67	0.50	75.00
M3							
AVPV	1	1	0	0	0.5	0.5	
rPVpo	0	0	0	0	0.0	0.0	
cPVpo	1	1	0	0	0.5	0.5	
Mean					0.33	0.33	100.00
M4							
AVPV	0	0	0	0	0.0	0.0	
rPVpo	0	0	0	0	0.0	0.0	
cPVpo	0	0	0	0	0.0	0.0	
Mean					0.00	0.00	0.00
M5							
AVPV	0	0	0	0	0.0	0.0	
rPVpo	0	0	0	0	0.0	0.0	
cPVpo	3	3	0	0	1.5	1.5	
Mean					0.50	0.50	100.00

Comparison of Percentage of Double-Labeled Kisspeptin Neurons Expressing AR or ERα in RP3V Region of Male Mice

The results of this study showed that percentage of kisspeptin neurons expressing AR is higher than the percentage of kisspeptin neurons expressing ERα (Figure 3.3), but the difference was statistically non-significant ($p<0.05$).

Co-localization of Kisspeptin and ERα in RP3V Region of Adult Female Mice

Kisspeptin and ERα double-labeled chromogen immunocytochemistry in the RP3V region of female mice showed the existence of many double-labeled cells as shown in Figure 3.4. Individual and mean number of single and double-labeled kisspeptin neurons in three regions of RP3V in female mice has been shown in Table 3.3. The percentage of double-labeled kisspeptin neurons in RP3V was 37.4 ± 5.3. Mean ± SEM number of kisspeptin neurons in RP3V region was 40.2 ± 5.6.

Co-localization of Kisspeptin and AR in RP3V Region of Adult Female Mice

Double-labeled chromogen immunocytochemistry showed the presence of many double-labeled kisspeptin cells (brown) with AR (black) in female mice (Figure 3.5) at three levels of RP3V. Individual and mean number of single and double-labeled kisspeptin cells has been shown in Table 3.4. The percentage of double-labeled kisspeptin neurons with AR in RP3V was 11.9 ± 2.1. Mean ± SEM number of kisspeptin neurons was 33.06 ± 9.4.

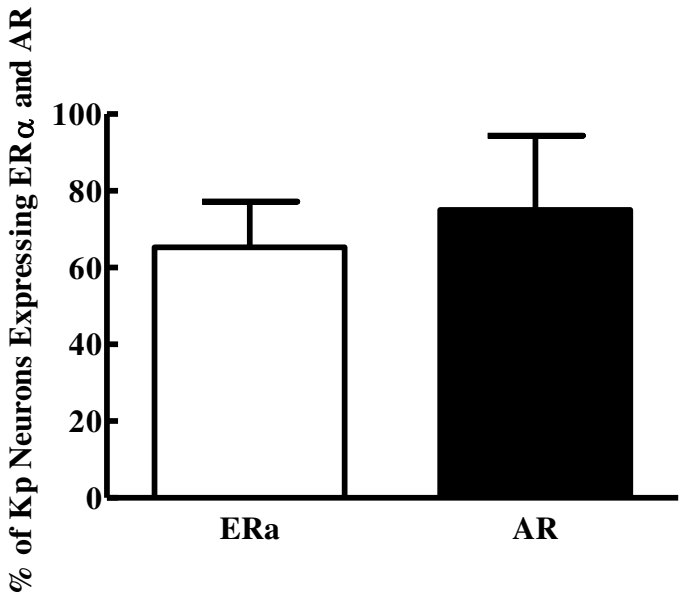

Figure 3.3. A bar graph indicating the mean ± SEM percentage of double-labeled kisspeptin neurons expressing ERα or AR in RP3V region of male mice (n=5).

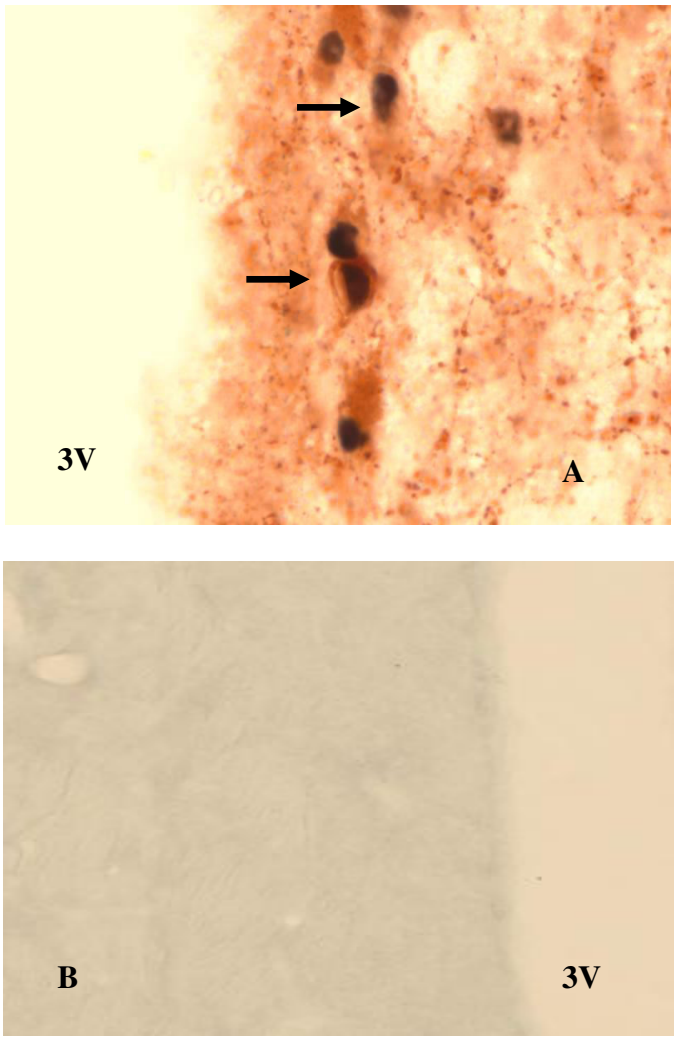

Figure 3.4. Representative photographs showing double chromogen labeling for kisspeptin neurons (brown) and ERα (black) in RP3V region of female mice at 600x magnifications (**A**) and control (**B**). "3V" denotes the third ventricle. Kisspeptin immunoreactivity was performed by treating with DAB and ERα immunocytochemistry was performed by treating with NiDAB. Arrows indicate dual-labeled kisspeptin cells with ERα.

Table 3.3. Individual and mean number of single (kp) and double-labeled (dbl) kisspeptin neurons expressing ERα observed in two sections from three regions of RP3V in female mouse brain

Animal No.	Section1		Section2		mean kp/ section	mean dbl/ section	%dbl
	kp	dbl	kp	dbl			
F1							
AVPV	20	13	33	17	26.5	15.0	
rPVpo	49	18	58	23	53.5	20.5	
cPVpo	72	13	61	13	66.5	13.0	
Mean					48.83	16.17	33.12
F2							
AVPV	16	10	38	21	27.0	15.5	
rPVpo	41	20	49	23	45.0	21.5	
cPVpo	50	18	47	20	48.5	19.0	
Mean					40.17	18.67	40.47
F3							
AVPV	12	7	30	20	21.0	13.5	
rPVpo	65	34	66	33	65.5	33.5	
cPVpo	60	24	79	40	69.5	32.0	
Mean					52.00	26.33	50.64
F4							
AVPV	40	16	37	15	38.5	15.5	
rPVpo	29	7	48	9	38.5	8.0	
cPVpo	35	10	30	5	32.5	7.5	
Mean					36.50	10.33	28.31
F5							
AVPV	21	8	0	0	10.5	4.0	
rPVpo	36	11	34	10	35.0	10.5	
cPVpo	51	11	0	0	25.5	5.5	
Mean					23.67	6.67	28.17

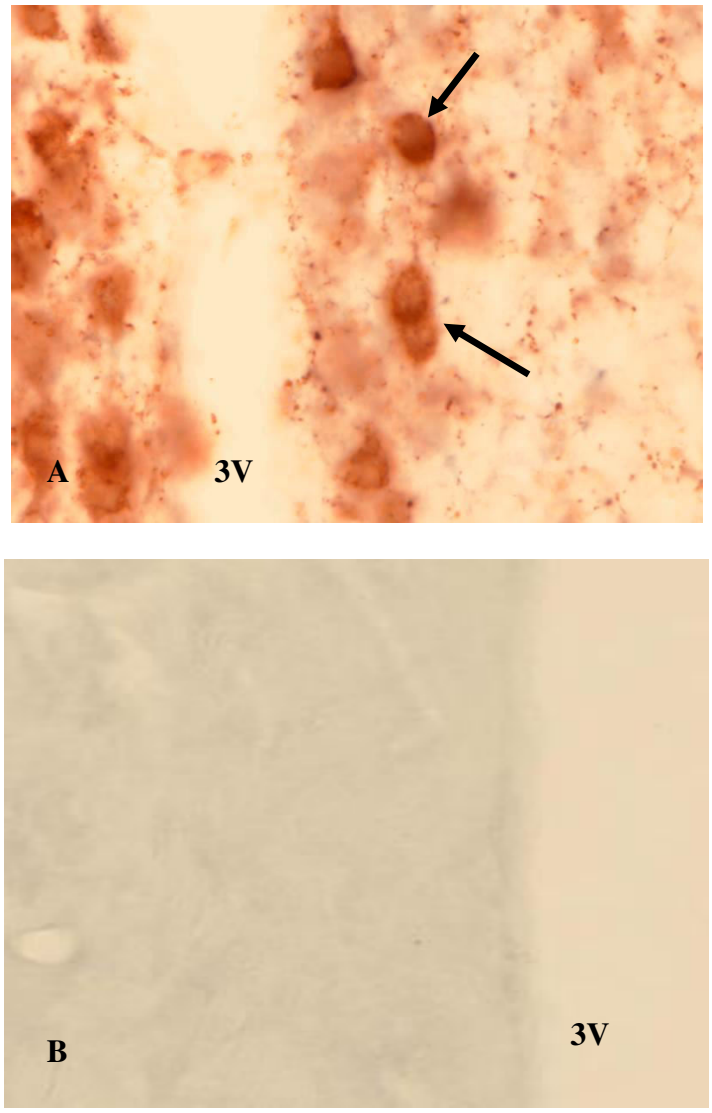

Figure 3.5. Representative photographs showing double chromogen labeling of kisspeptin neurons (brown) and AR (black) in the RP3V region of female mice at 600x magnification (**A**) and control (**B**). "3V" denotes the third ventricle. Kisspeptin immunoreactivity was performed by treating with DAB and AR immunocytochemistry was performed by treating with NiDAB. Arrows indicate dual-labeled kisspeptin cells with AR.

Table 3.4. Individual and mean number of single (kp) and double-labeled (dbl) kisspeptin neurons expressing AR observed in two sections from three regions of RP3V in female mouse brain

Animal No.	Section1		Section2		mean kp/ section	mean dbl/ section	%dbl
	kp	dbl	kp	dbl			
F1							
AVPV	18	0	15	0	16.5	0.0	
rPVpo	21	4	30	8	25.5	6.0	
cPVpo	10	1	12	2	11.0	1.5	
Mean					17.67	2.50	14.15
F2							
AVPV	15	3	45	7	30.0	5.0	
rPVpo	35	3	51	7	43.0	5.0	
cPVpo	32	4	17	2	24.5	3.0	
Mean					32.50	4.33	13.33
F3							
AVPV	25	1	27	6	26.0	3.5	
rPVpo	43	4	36	8	39.5	6.0	
cPVpo	8	1	15	3	11.5	2.0	
Mean					25.67	3.83	14.94
F4							
AVPV	13	1	27	2	20.0	1.5	
rPVpo	33	6	30	5	31.5	5.5	
cPVpo	23	2	18	2	20.5	2.0	
Mean					24.00	3.00	12.50
F5							
AVPV	40	4	46	2	43.0	3.0	
rPVpo	93	3	88	4	90.5	3.5	
cPVpo	70	2	56	3	63.0	2.5	
Mean					65.50	3.00	4.60

Comparison of Percentage of Double-Labeled Kisspeptin Neurons Expressing ERα or AR in RP3V of Female Mice

The results of this study showed that the percentage of kisspeptin neurons expressing ERα was significantly ($p<0.01$) higher than the percentage of kisspeptin neurons expressing AR (Figure 3.6).

Comparison of Percentage of Double-Labeled Kisspeptin Neurons Expressing ERα or AR between Male and Female Mice

The overall percentage of double-labeled kisspeptin neurons expressing androgen receptors was significantly ($P<0.05$) higher in male mice as compared to female mice (Figure 3.7A). Similarly, the percentage of kisspeptin neurons expressing ERα was also higher in male mice as compared to female mice (Figure 3.7B) but this elevation in percentage was statistically non-significant.

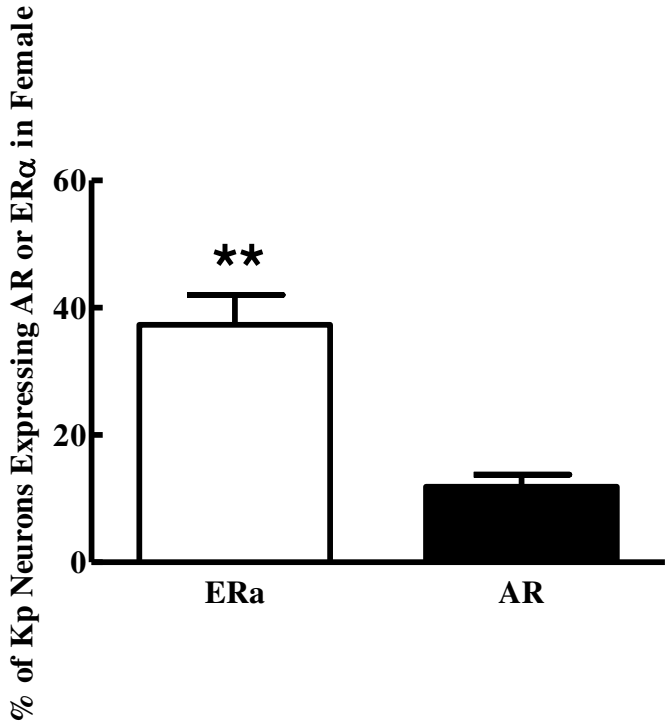

Figure 3.6. A bar graph showing the mean ± SEM percentage of double-labeled kisspeptin neurons expressing ERα or AR in RP3V region of female mice (n=5). Percentage of ERα expressing kisspeptin cells was significantly (**p<0.01) greater than AR expressing kisspeptin cells in female mice.

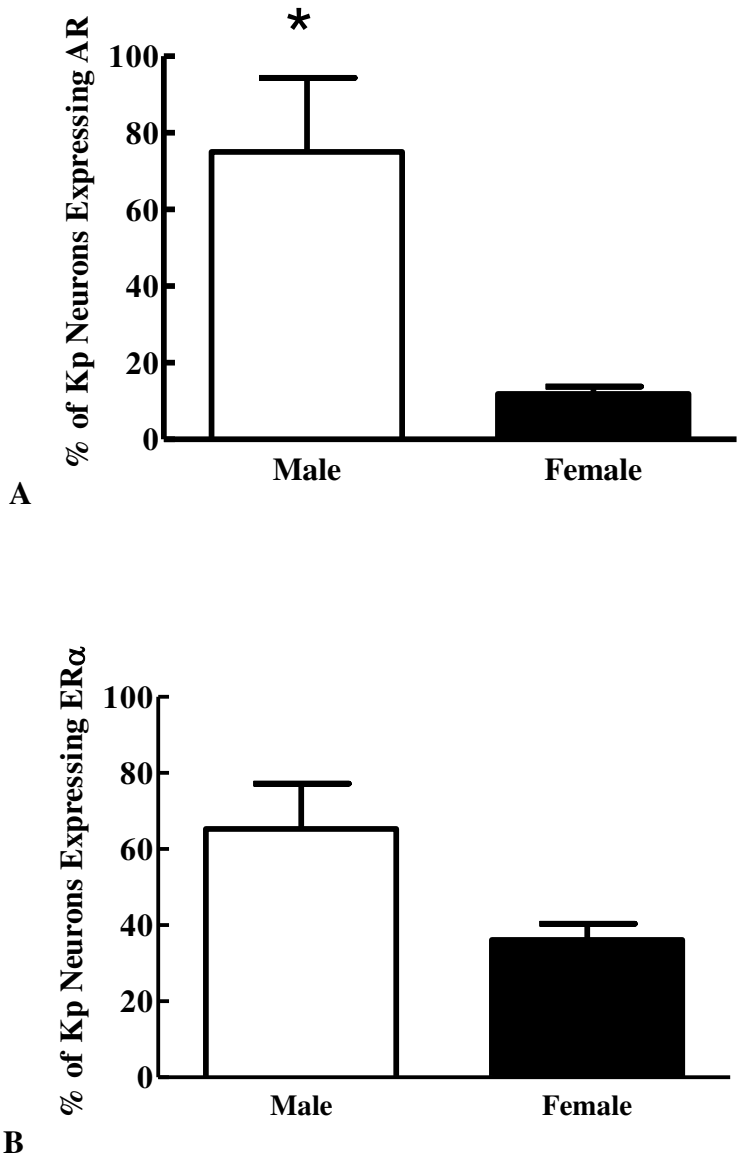

Figure 3.7. A bar graph showing the mean ± SEM percentage of double-labeled kisspeptin neurons expressing AR (**A**) and ERα (**B**) in RP3V region of male and female mice (n=5). Percentage of AR expressing kisspeptin cells was significantly (*p<0.05) greater in male mice as compared to female mice (**A**).

DISCUSSION

Following the pioneer observations in 2003 that mutations of *KISS1R* and *Kiss1r* in humans and mice, respectively, result in hypogonadotropic hypogonadism and failure in puberty (de Roux et al., 2003; Seminara et al., 2003), many experiments were done to study the role of kisspeptin signaling in the regulation of reproduction. Almost all of the attention has been paid to analyse the kisspeptin neurons and their role in GnRH regulation. However, the regulation of kisspeptin neurons in general and more specifically, in the RP3V has been ignored. Hitherto gonadal steroids have been revealed to be important regulators of kisspeptin neurons in mice and rats (Smith et al., 2005a; 2005b). Here, we show that AR and Erα are expressed by kisspeptin neurons in both male and female mice.

Kisspeptin neurons exist in AVPV and PVpo regions known as RP3V in mouse brain (Clarkson et al., 2009). Several studies reported that the RP3V population of kisspeptin neurons directly interacts with GnRH neurons (Clarkson et al., 2008). In this study, RP3V population of kisspeptin neurons was selected to examine the co-localization of kisspeptin neurons and AR/ERα as this population is activated at the time of GnRH/LH surge in rodents (Smith et al., 2008; Clarkson et al., 2008). In contrast, ARC kisspeptin neuronal population does not activate at the time of GnRH/LH surge and can be identified as early as embryonic d 12 in the mouse (Knoll et al., 2008) and does not show any developmental change across the postnatal period (Han et al., 2005; Kauffman et al., 2007).

Our findings showed that the majority of kisspeptin neurons of adult male and female mice express ERα and AR in RP3V region. Overall 65% and 37% of kisspeptin neurons expressed ERα in RP3V in adult male and female mice, respectively. Our

data established that kisspeptin neurons express ERα in both male and female mice. In female mouse ERα are involved in positive feedback regulation by estrogen, which is involved in preovulatory LH/GnRH surge (Christian et al., 2008). As previously reported, effect of estradiol on the *Kiss1* mRNA appeared to be mediated by ERα because *Kiss1* expression was unresponsive to estradiol treatment in mice with genetically targeted deletions of this ER isoform (Smith et al., 2005a). However, the role of ERα expressed on kisspeptin neurons in RP3V in male mouse is unknown. We speculate that ERα might be involved in mediating testosterone actions on RP3V kisspeptin neurons. In mallard drake, kisspeptin cells express aromatase (Saldanha et al., 2010). Conversion of testosterone to estradiol by aromatase (Meisel and Sachs, 1994) and its subsequent signaling through estrogen receptor (Gustafsson et al., 1999) has been well established. It has been known that ERα knockout (ERαKO) mice are infertile due to changes in testicular picture as revealed by atrophy of seminiferous tubules, reduction in sperm count, failure to copulate and impaired sexual behaviour (Eddy et al., 1996; Rissman et al., 1997; Wersinger et al., 1997; Wersinger and Rissman, 2000). It is likely that these ERαKO mice lack ERα on kisspeptin cells. This observation depicts some stimulatory action of steroids on RP3V kisspeptin cells as previously observed by Smith et al. (2005b) in AVPV of male mice. Positive feedback pathway of steroids to GnRH mediated through RP3V kisspeptin cells in male mice might be a remnant of positive feedback circuitry that exists in female and its function in male mice is vestigial. There is also a possibility that the RP3V kisspeptin population serve other testosterone dependent functions such as sexual behaviour. These findings suggest the possible involvement of ERα expressing RP3V kisspeptin neurons in the sexual behaviour of male mice.

Moreover, we observed that kisspeptin neurons in AVPV were apparently sexually differentiated, with the female AVPV having far more kisspeptin neurons than the male. As noted previously that the AVPV is sexually dimorphic nucleus and plays an imperative role in preovulatory GnRH/LH surge (Simerly, 1998; Gu and Simerly 1997). This notion is further supported by finding that AVPV *Kiss1* mRNA expression was far greater in female than in male (Smith et al., 2005b).

Results of our investigations show that RP3V kisspeptin neurons also expressed AR. About 75% and 11.9% of kisspeptin neurons express AR in RP3V of adult male and female mice, respectively. In a recent study, an evidence has been found that steroids regulate the *Kiss1* expression in MeA region in both male and female rodents. This steroid regulation of *Kiss1* occurs through estrogen receptor dependent pathways (Kim et al., 2011). In male mouse, ARC-kisspeptin neurons mediate negative feedback effect of testosterone to GnRH secretion whereas; RP3V kisspeptin neurons may mediate positive feedback effects of steroids to other testosterone dependent processes (Smith et al., 2005b).

Testosterone stimulates *Kiss1* mRNA expression in the AVPV of male mice (Smith et al., 2005b). But the physiological significance of this testosterone stimulation of RP3V kisspeptin neurons of male rodents is unknown. It has been well established that RP3V is involved in the regulation of GnRH secretion particularly, in pre-ovulatory LH surge in female mouse (Simerly, 1998; Gu and Simerly, 1997). Thus, RP3V kisspeptin neurons mediate estrogen dependent positive feedback effects of estradiol in female mice but their physiological significance in male is uncertain. In male mouse, RP3V population of kisspeptin neurons may serve other testosterone dependent functions such as sexual behaviour. The RP3V has been found to be involved in the control of sexual behaviour (Simerly, 1998). RP3V receives sensory

information from BnST and medial amygdala and then sends this information to the POA (Simerly, 2002). All of these components are involved in sexual behaviour of male (Paredes and Baum, 1997). There is an evidence that a rich population of dopaminergic neurons is present in the RP3V region (Simerly et al., 1997) and a subpopulation of kisspeptin neurons express dopamine (Clarkson and Herbison, 2011), which has been involved in the regulation of male sexual behaviour (Dominguez and Hull, 2005). On the basis of these findings, we speculate that the RP3V population of kisspeptin neurons in male mouse might be involved in the mediation of testosterone associated effects on sexual behaviour.

Our findings show that percentage of AR expressing kisspeptin neurons tend to be higher than kisspeptin cells expressing ERα in male mice. On the other hand, percentage of ERα expressing kisspeptin neurons was significantly greater in female mice as compared to kisspeptin cells expressing AR. We also observed that 65% of RP3V kisspeptin cells expressed ERα and 75% of RP3V kisspeptin cells expressed AR. This observation suggests the possibility of the existence of kisspeptin cells expressing both ERα and AR. This finding also suggests the existence of fine resolution of steroid regulation of RP3V kisspeptin cells in male mice. In addition, these findings suggest that in male mice both androgen and estrogen receptors on RP3V kisspeptin neurons are equally important in mediating steroid effects to GnRH neurons as observed during pubertal development in the male mouse (Clarkson et al., 2012). We can also speculate that steroid receptors present on RP3V kisspeptin may regulate male sexual behaviour in adult male mice as previously observed by Smith et al. (2005b). It has been well established in female mice that ERα expressing RP3V kisspeptin cells are involved in mediating positive feedback effects of steroids in inducing preovulatory LH surge (Christian et al., 2008). However, the role of AR

expressing RP3V kisspeptin cells in female mice is uncertain. It is imaginable that AR may contribute to a smaller extent in mediating positive feedback effects of steroids to preovulatory LH surge in female mice. Thus, both androgen and estrogen receptors are likely to be important in the regulation of sexual behaviour in male mice and estrogen receptors are important for inducing preovulatory LH surge in female mice.

We summarize that in addition to previously documented expression of ERα by kisspeptin neurons in female mice, ERα are also expressed by kisspeptin neurons in male mouse. We provide evidence that AR receptors are also expressed by kisspeptin neurons in RP3V region of both male and female mice. Our data suggest that androgen and estrogen receptors are involved in the regulation of GnRH secretion and sexual behaviour during adulthood or a dormant positive feedback circuitry in male mice. On the other hand, in female mice AR may contribute in mediating steroid positive feedback effects to induce a pre-ovulatory LH surge. We suggest that both androgen and estrogen receptors are involved in the steroid regulation of kisspeptin neurons in male and female rodents.

General Discussion

GENERAL DISCUSSION

In the research work for the present dissertation, we studied the neurotransmitter (glutamate and GABA) regulation of kisspeptin neurons in adult male rhesus macaque during different reproductive seasons and metabolic states. Additionally, we studied the expression of steroid receptors on specific population of kisspeptin neurons to better understand the steroids regulation of kisspeptin neurons in adult male and female mice. We achieved our objectives by performing different experiments. Firstly, we performed dual label immunofluorescence for kisspeptin and NR1 subunit of NMDA receptors, and compared and quantified *Kiss1, Kiss1r, NR1* and *GAD67* mRNA expression levels in MBH and POA of the adult male rhesus macaques, during the breeding and non-breeding season. Secondly, we performed dual label immunofluorescence for kisspeptin and NR1 and quantified and compared *Kiss1, Kiss1r, NR1* and *GAD67* mRNA expression levels in MBH and POA of the adult male rhesus monkey during normal feeding and 48 h fasting states. Plasma levels of testosterone were also assessed to demonstrate changes occurring in the reproductive axis during different reproductive seasons and metabolic states in monkeys. Thirdly, we performed dual label chromogen immunocytochemistry for kisspeptin neurons and androgen/estrogen receptors in the RP3V region of both male and female mice.

Overall our results demonstrate that kisspeptin neurons are regulated by excitatory and inhibitory neurotransmitters accompaniments of environmental and metabolic cues in rhesus macaques and by steroids in mice. In first set of experiments, we tried to decipher the function of excitatory (glutamate) and inhibitory (GABA) neurotransmitters in the regulation of kisspeptin signaling in adult male rhesus macaques during the breeding and non-breeding season, physiological scenarios

where reproductive axis is distinctly modulated. We achieved our objective by quantifying and comparing *Kiss1, Kiss1r, NR1* and *GAD67* mRNA contents in MBH and POA and by performing dual label immunofluorescence of kisspeptin neurons and NR1 in MBH of adult male monkeys during different reproductive states. Results of this study demonstrated that kisspeptin signaling increased during the breeding season as compared to the non-breeding season which was revealed by the increase in kisspeptin peptide expression and *Kiss1* and *Kiss1r* mRNA levels in MBH and POA. Results of this study also demonstrated that increased kisspeptin signaling during the breeding season was due to increase in glutamatergic inputs to kisspeptin neurons as revealed by the increase in percentage of NR1 expressing kisspeptin neurons and *NR1* mRNA contents, and decrease in GABA synthesis as shown by the decrease in *GAD67* mRNA levels. Plasma testosterone levels and testicular volume were also elevated during the breeding season. It has been found recently that kisspeptin neurons receive cues from afferent excitatory and inhibitory neurotransmitters (d'Anglemont de Tassigny et al., 2010; Kurian et al., 2012). Our findings also suggest that glutamate signaling increases whereas GABA signaling decreases during the breeding season as compared to the non-breeding season. Our results are in accordance with the previous findings that glutamatergic signaling increased whereas GABAergic signaling decreased during the breeding season as compared to non-breeding season in ewe (Sergeeva et al., 2009). This increase in glutamate drive and decrease in GABA activity in the hypothalamus of adult male monkeys during the breeding season are most likely to cause the stimulation of kisspeptin signaling.

The foregoing notion is supported by the evidence that central NMDA administration causes increase in c-fos expression in kisspeptin cells (d'Anglemont de Tassigny et al., 2010). This finding suggests that glutamate may stimulate kisspeptin neurons.

Similarly, administration of GABA antagonist (bicuculline) in pre-pubertal monkeys arouses kisspeptin release (Kurian et al., 2012). Some electrophysiological studies also revealed a direct interaction between kisspeptin and glutamate/GABA. It is recently observed that ARC-KNDY neurons and RP3V-Kiss1 neurons are stimulated by NMDA and inhibited by GABA (Ducret et al., 2010; Gottsch et al., 2011; Qiu et al., 2011; Kurian et al., 2012).

Results of the current research work suggest that kisspeptin signaling increased during breeding season in primates. This finding is consistent with the report of Smith et al. (2007) which demonstrated that in short day breeders such as ewe, *Kiss1* mRNA expression increased during short days. Similarly, number of kisspeptin neurons in the ARC and POA of the ewe brain are increased when they are shifted to short day environment from long day conditions, and vice versa (Chalivoix et al., 2010). However, in long day breeders such as Syrian hamsters, *Kiss1* mRNA levels in AVPV increased during long days (Paul et al., 2009). In the same way, *Kiss1* mRNA contents in the ARC region of male Syrian hamsters are decreased when they are shifted to short day environment from long day conditions (Revel et al., 2006). This decrease in *Kiss1* mRNA expression after shifting to short day environment seems to be dependent on melatonin because reduction in kisspeptin expression in Syrian hamsters after short day treatment is inhibited by pineal gland ablation (Revel et al., 2006). Later finding indicates that seasonal cues to kisspeptin signaling are mediated by melatonin. It is unclear whether melatonin acts on kisspeptin neurons directly through melatonin receptors or through intermediate pathways in Syrian hamsters. However, kisspeptin neurons do not express melatonin receptors in ewe suggesting the indirect effects of melatonin on kisspeptin (Li et al., 2011). Whether kisspeptin neurons express melatonin receptors in monkeys have not been examined so far. There are

some studies which showed that melatonin may interact with glutamate and GABA in rats (Marquez de Prado et al., 2000). Melatonin perfusion in neostriatum of rats, suggests some stimulatory effects of melatonin on glutamate and no effects on GABA (Marquez de Prado et al., 2000). Furthermore, melatonin receptor type 1 (MT1) has also been co-localized with glutamatergic neurons in dorsal habenula neurons of transgenic mouse expressing red fluorescent protein at MT1 receptor promoter (Evely et al., 2013). Similarly, GABAergic neurons localized in retina also express MT1 receptors in guinea pig (Fujieda et al., 2000). On the basis of these observations we suggest that melatonin signal may regulate glutamate and GABA signaling in monkey hypothalamus which in turn modulates kisspeptin signaling.

Taken together the results of our this study suggest that increased kisspeptin signaling during the breeding season is due to increased glutamate action and decreased GABA levels in monkey hypothalamus. This was evident by the increase in *Kiss1, Kiss1r* and *NR1* mRNA levels, and decrease in *GAD67* mRNA levels in the MBH and POA of adult male rhesus macaque during the breeding season. Similarly, kisspeptin peptide expression and the number of NR1 expressing kisspeptin neurons were also increased in MBH of adult male monkeys during the breeding season. These findings led us to conclude that NMDA and GABA operate in concert, with complementary effects, to control kisspeptin signaling in rhesus macaque across the season, leading to yearly change in fertility.

Since neurotransmitter regulation of kisspeptin neurons was evident in adult male rhesus macaque across the season, we also studied the regulation of kisspeptin signaling during different metabolic states in adult male monkeys. Changes in energy metabolism have been observed to effect HPG axis in a host of species including mammals, primates and humans (Bronson, 1986; Armstrong and Britt, 1987;

Bronson, 1988; Cameron and Nosbisch, 1991; Schreihofer et al., 1993; Cameron, 1996; Lado-Abeal et al., 1999). In our second set of experiments, we found that fasting for 48 h in intact adult male rhesus monkey induced suppression of HPG axis, which was evident by a significant decrease in plasma testosterone concentrations. Blood glucose levels were also significantly decreased after short term fasting, it is in line with the previous studies in many species such as ewes, pigs, monkeys and humans (Heitmann et al., 1986; Beer et al., 1989; Fairhall et al., 1990; Barb et al., 2002). Our immunocytochemistry data showed that kisspeptin peptide expression and its interaction with NR1 subunit decreased after 48 h of food deprivation. Our PCR data showed a decrease in *Kiss1* and *Kiss1r* mRNA levels after 48 h of fasting along with a decrease in *NR1* and unaltered *GAD67* expression.

Our findings of decrease in *Kiss1* and *Kiss1r* in fasting are consistent with the previous observation in male monkeys (Wahab et al., 2010). Similarly, decrease in expression of *NR1* mRNA after 48 h of fasting is in accordance with the previous finding, by Shahab et al. (1997), in which testosterone response to NMDA administration was lesser in monkeys subjected to short term fasting as compared to the monkeys fed *ad libitum*. This suggests that short term fasting decreases the NMDA receptor expression or sensitivity to exogenous NMDA administration in adult male monkeys.

Our results of this study demonstrated that fasting induced suppression of hypothalamic kisspeptin signaling is due to the decrease in excitatory inputs to kisspeptin neurons. Another important finding of this study was that GABAergic system was not involved in fasting induced suppression of HPG axis and kisspeptin signaling in male monkeys, since *GAD67* mRNA content remained unchanged after 48 h of fasting. It suggests that fasting for 48 h in intact adult male monkeys does not

cause any increase in inhibitory GABA based inputs to kisspeptin neurons, which is revealed by the constant expression of *GAD67* mRNA levels in hypothalamus. Our data is in line with the previous observations in male Wistar rats, which showed that *GAD67* mRNA expression was not changed after short term fasting (Schwartz et al., 1993). However, in contrast to our findings, Leonhardt et al. (1999) reported that food restriction in male rats caused an increase in the activity of GABAergic neurons, which then mediated inhibitory effect of fasting on the HPG axis. The reason for this difference could be due to the difference in experimental model, as Leonhardt et al. (1999) used male rats while we used intact adult male monkeys. Secondly, there was a difference in the nature of metabolic treatment i. e. fasting verses food restriction. Another reason could be the difference in the duration of food deprivation, as above mentioned study applied food deprivation for 17-days, while in the present study we subjected the monkeys for 48 h of fasting condition. GABA receptor expression and its sensitivity were not examined in this study. It is possible that GABA receptor sensitivity may be modulated during short term fasting and may contribute to suppression of kisspeptin signaling in fasting. Such a notion appears possible in view of previously observed involvement of GABA receptor sensitivity changes in causation of pre-pubertal hiatus of GnRH secretion in female monkeys (Mitsushima et al., 1994; 1996). In summary, the results of our this study suggest that fasting induced suppression of kisspeptin signaling is mediated through excitatory neurotransmitters and not through the inhibitory neurotransmitters in adult male rhesus macaque.

The fact that kisspeptin signaling is regulated by excitatory and inhibitory neurotransmitters in adult male rhesus monkeys during different reproductive seasons and metabolic states is evident by our data. In addition to neurotransmitter regulation of kisspeptin neurons in higher primates there is strong evidence that estrogens or

androgens are involved in the steroidal regulation of ARC kisspeptin neurons. However, such a steroid regulation of kisspeptin neurons present in RP3V is less clear. Therefore, next we tried to assess the expression of androgen and estrogen receptors on RP3V population of kisspeptin neurons in adult male and female mice. Our results of this study suggest that RP3V kisspeptin neurons express both estrogen and androgen receptors in male and female mice. This shows the importance of both estradiol and testosterone in the regulation of kisspeptin neurons in adult male and female mice. In female mice, ERα expressing RP3V kisspeptin neurons are involved in mediating positive feedback effects of estradiol to induce preovulatory LH/GnRH surge (Smith et al., 2006; Adachi et al., 2007; Clarkson et al., 2008). Although, such a positive feedback regulation by estradiol to stimulate LH/GnRH secretion is absent in male mice, kisspeptin signaling is important for the onset of puberty and fertility in males as demonstrated by lack of puberty onset and fertility in mice lacking GPR54 (Funes et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007). Recently, it has been reported that steroid hormones are important in the pubertal increase in the number of kisspeptin neurons in RP3V of male (Clarkson et al., 2012) and female mice (Clarkson et al., 2009b; Mayer et al., 2010). Similarly, removal of the testes or ovaries before pubertal development causes a reduction in the number of kisspeptin neurons in RP3V of male and female mice at puberty (Bakker et al., 2009; Clarkson et al., 2009b; Mayer et al., 2010; Clarkson et al., 2012). This finding suggests the importance of gonads and steroids in the stimulation of RP3V kisspeptin neurons during pubertal development.

It has been reported recently that estradiol acting through ERα is critical for the pubertal rise in the expression of kisspeptin in female mice (Mayer et al., 2010). Our results demonstrated that the majority of RP3V kisspeptin neurons expressed ERα and

AR in adult male and female mice. Similar to our findings, earlier studies demonstrated the co-expression of *Kiss1* and ERα in male and female mice (Smith et al., 2005a, b). Percentages of RP3V kisspeptin neurons expressing ERα in male and female mice observed in our study were statistically similar. In female mice, role of ERα expressing RP3V kisspeptin cells in stimulation of preovulatory LH/GnRH surge has been well established. However, the function of ERα on this particular kisspeptin neuronal population in male mice is uncertain. One can expect that ERα might be involved in mediating the testosterone action on RP3V kisspeptin neurons. It has been well known that testosterone converts into estradiol by aromatase (Meisel and Sachs, 1994) and its subsequent signaling occurs through estrogen receptor (Gustafsson et al., 1999). It has also been found recently that kisspeptin cells express aromatase in mallard drake (Saldanha et al., 2010). It has been known that ERα knockout (ERαKO) mice are infertile due to changes in the testicular picture as revealed by atrophy of seminiferous tubules, reduction in sperm count, failure to copulate and impaired sexual behaviour (Eddy et al., 1996; Rissman et al., 1997; Wersinger et al., 1997; Wersinger and Rissman, 2000). It is likely that these ERαKO mice lack ERα on kisspeptin cells.

Together foregoing observations and assumptions would suggest some stimulatory action of estradiol on RP3V kisspeptin cells, as previously observed by Smith et al. (2005b) in the AVPV of male mice. However, the exact role of this estradiol mediated positive feedback circuitry in male mice is not clear. It is possible that this positive feedback pathway of estradiol to GnRH may be a remnant of positive feedback circuitry that exists in female mice and that its function in male mice is vestigial. Such a notion is supported by the novel observation that this estradiol induced LH surge like pattern can be demonstrated in male monkeys (Norman and Spies, 1986). It is

imaginable that ERα present on RP3V kisspeptin cells may play a role in the sexual behaviour, pubertal increase in the kisspeptin neuronal number and may regulate a dormant estradiol mediated positive feedback pathway in male mice.

Our findings of this study also showed that percentage of RP3V kisspeptin neurons expressing AR was significantly higher in male mice as compared to female mice. In female mice, the source of testosterone is likely to be the ovaries, as rats and mice lack the enzyme 17 alpha-hydroxylase necessary to synthesize adrenal androstenedione and dehydroepiandrosterone (DHEA). Therefore, the adrenals in these animals do not contribute to plasma levels of DHEA and androstenedione (Van Weerden et al., 1992). However, the role of AR present on RP3V kisspeptin cells in female mice is uncertain. It is likely that AR may contribute to a smaller extent in mediating positive feedback effects of testosterone to preovulatory LH surge in female mice. It has been well established that in male mice, kisspeptin neurons in the ARC region mediate negative feedback effects of testosterone to GnRH secretion whereas RP3V kisspeptin neurons mediate positive feedback effects of steroids (Smith et al., 2005b) which have been suggested to mediate other testosterone dependent processes such as sexual behaviour (Smith et al., 2005b). However, the physiological importance of this AR expressing RP3V kisspeptin neuronal population in male mouse is unknown. It is likely that AR expressing RP3V population of kisspeptin cells in male mice may serve other functions, such as sexual behaviour, which is stimulated by testosterone. Although in mice, appropriate kisspeptin signaling during the perinatal period is obligatory for the development of proper sexual behaviour in adulthood (Kauffman et al., 2007b), the role of kisspeptin signaling in adults needs future studies.

Parenthetically, RP3V has been involved in the control of sexual behaviour (Simerly, 1998). RP3V receives sensory information from medial amygdala and BnST and then sends this information to the POA (Simerly, 2002), and so all of these have been recognized to be involved in affecting the male sexual behaviour (Paredes and Baum, 1997). There is also the evidence that in RP3V region, a subpopulation of kisspeptin neurons express dopamine (Clarkson and Herbison, 2011), and it is also recognized to be involved in regulating male sexual behaviour (Dominguez and Hull, 2005).

On the basis of these findings, we propose that the stimulation of the RP3V population of kisspeptin neurons by testosterone in male mouse may be involved in mediating testosterone's effects on sexual behaviour. It is imaginable that AR present on RP3V kisspeptin neurons may mediate positive feedback effects of testosterone to regulate sexual behaviour, pubertal increase in kisspeptin neurons or control a vestigial pathway in male mice and play a role in the preovulatory LH surge in female mice.

Our results demonstrate that there was differential expression of ERα and AR on kisspeptin neurons in the RP3V region in male and female mice. Male mice showed statistically similar number of ERα and AR expressing RP3V kisspeptin cells whereas female mice had significantly higher number of ERα expressing kisspeptin cells as compared to AR expressing kisspeptin cells. We observed that 65% of RP3V kisspeptin cells expressed ERα and 75% of RP3V kisspeptin cells expressed AR in male mice. On the basis of this observation, we suppose that there may be one population of kisspeptin cells which expresses both ERα and AR. This observation suggests the existence of fine resolution of steroid regulation of RP3V kisspeptin cells in male mice. Further, these findings also suggest that in male mice both androgen and estrogen receptors on RP3V kisspeptin neurons are equally important in

mediating steroid effects to GnRH neurons as previously observed during pubertal development in the male mouse (Clarkson et al., 2012). Our results also suggest that both testosterone and estradiol are equally important in the regulation of RP3V kisspeptin neuronal population. On the other hand, 37% of RP3V kisspeptin cells expressed ERα and 11.9% of RP3V kisspeptin cells expressed AR in female mice. On the basis of this observation it is less likely that some RP3V kisspeptin neurons express both AR and ERα in female mice but this option cannot be ruled out.

Our results also indicated significant higher prevalence of ERα as compared to AR in RP3V kisspeptin neurons in females. This finding suggests that ERα are more important than AR. However, the role of AR expressing RP3V kisspeptin cells in female mice is uncertain. It is imaginable that AR may contribute to a smaller extent in mediating positive feedback effects of steroids to preovulatory LH surge in female mice. Thus, both androgen and estrogen receptors are likely to be important in the regulation of kisspeptin neurons during sexual behaviour and pubertal increase of kisspeptin neurons in male mice. While estrogen receptors are more important for stimulation of RP3V kisspeptin cells leading to induce preovulatory LH surge in female mice.

In the current study we observed the changes in the expression of genes of kisspeptin, kisspeptin receptor, NMDAR1 and GAD67 in the monkey hypothalamus during different seasons and metabolic states. Our PCR data about the correlative changes in gene expression is suggestive. However, our data of the immunofluorescence strengthen our PCR data and we can now conclude with better degree of confidence about the regulation of kisspeptin neurons by excitatory neurotransmitters in adult male macaques during different reproductive seasons and metabolic states. We recognize that we did not perform immunocytochemistry for kisspeptin and GABA

receptor in the current study which would have strengthened our gene expression data.

Taken together the results of different studies included in the present thesis suggest that kisspeptin signaling pathway is influenced by many internal and external cues. Kisspeptin signaling pathway is imperative for the regulation of HPG axis and hence reproduction. It is a strong stimulator of GnRH neuronal secretion. In addition, kisspeptin plays an imperative role in the maintenance of reproductive functions in adults. Data obtained in our first study suggest that increased kisspeptin signaling in hypothalamus of adult male rhesus macaque during breeding season is due to the increase in glutamate action and decrease in GABA based inhibitory inputs. In our second study, results imply that predominantly glutamate neurotransmission is involved in mediating short term fasting induced suppression of HPG axis and kisspeptin signaling in adult male monkeys. On the other hand, GABA is not found to be involved in fasting induced suppression of kisspeptin signaling and HPG axis. Finally, the results of our last experiment showed that a specific hypothalamic population of kisspeptin neurons is regulated by steroid hormones as revealed by differential expression of ERα and AR on kisspeptin neurons in male and female mice.

The results of the present research work strengthened our knowledge about the regulation of kisspeptin neurons by excitatory and inhibitory neurotransmitters and by steroids in monkeys and mice under different physiological conditions. Our results improved our knowledge about the neurotransmitter regulation of kisspeptin pathways especially in the hypothalamus of the higher primates. Present results have also contributed to the current understanding on steroid regulation of kisspeptin neurons in rodents by demonstrating the relative expression of estradiol and testosterone

receptors on RP3V population of kisspeptin neurons. However, complete elucidation of the regulation of kisspeptin signaling by these pathways in mice and monkeys is needed to be explored in future studies. Further work is needed to clarify the possible mechanisms by which GABA modulates kisspeptin signaling. In this respect it would be useful to study the expression of GABA receptors on kisspeptin neurons in primates during different physiological states. Likewise, role of factors produced during energy challenge and their interaction with glutamatergic and GABAergic pathways will be important to study. To fully understand the photoperiodic regulation of kisspeptin signaling, it is needed to locate the melatonin receptors on kisspeptin, glutamatergic and GABAergic neurons in primate hypothalamus. Regulation of kisspeptin signaling by different external and internal cues is an important segment in HPG axis. Elucidating the regulation of kisspeptin signaling would be helpful in resolving many reproductive health problems in humans and would lead to the development of strategies for regulating fertility and infertility in humans.

Overall, the findings of this research work strengthen our knowledge about the neurotransmitter regulation of kisspeptin neurons in the hypothalamus of adult male rhesus macaque during different physiological conditions. In addition our current understanding about the steroid regulation of kisspeptin neurons in RP3V region of both male and female mice has been enhanced by this investigation. Based on the findings of this dissertation we proposed a model about the regulation of kisspeptin neurons shown in Figure iii.

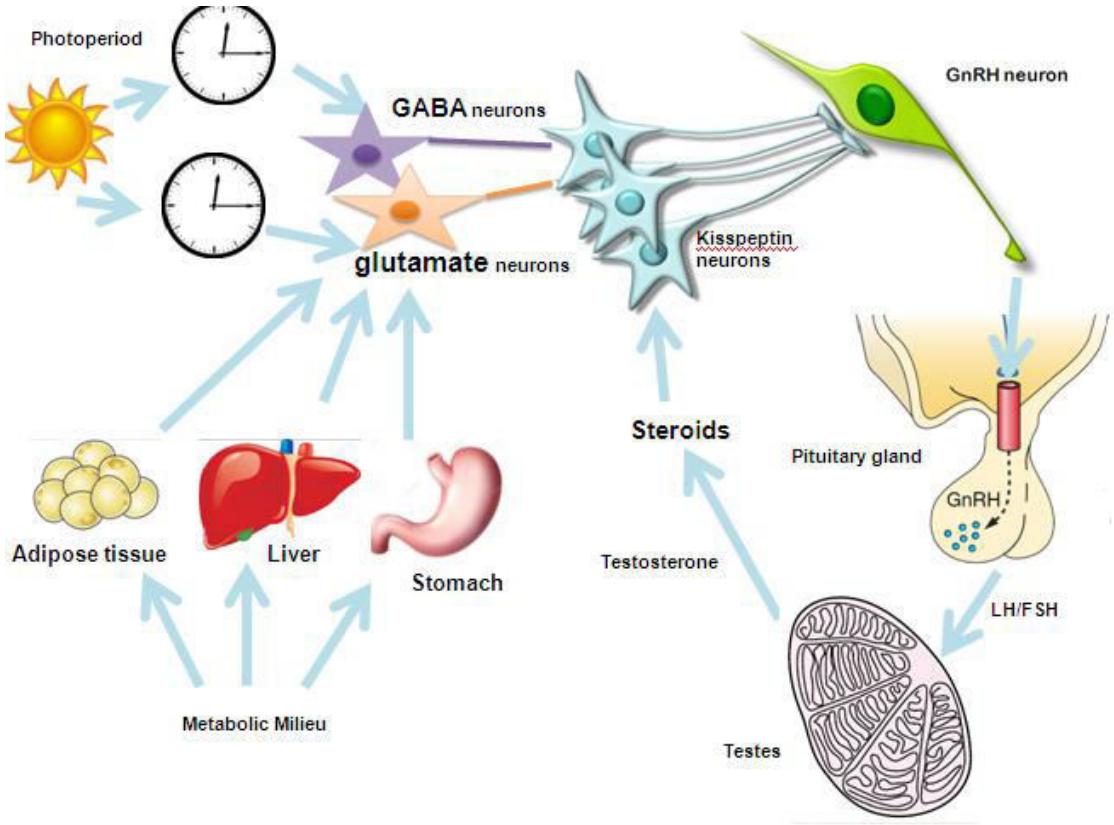

Figure iii. A proposed model for the neurotransmitter regulation of kisspeptin neurons in higher primates. Metabolic signals (adipose tissues, stomach, liver), gonadal steroids and photoperiods regulate kisspeptin neurons.

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