Studies of the Role of Kisspeptin-GPR54 Signaling in the Testis

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STUDIES OF THE ROLE OF KISSPEPTIN-GPR54 SIGNALING IN THE TESTIS

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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.

Amna Rashid Tariq

CERTIFICATE

The Thesis titled "Studies of the role of kisspeptin-GPR54 Signaling in the testis" submitted by **Ms. Amna Rashid Tariq** is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Doctor of Philosophy in Endocrinology.

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Amna Rashid Tariq

LIST OF ABBREVIATIONS

°C	Centigrade
µl/µL	Microliter
mM	Millimol
ACTB	ß-actin
AVPV	Anteroventral periventricular nucleus
ANOVA	Analysis of variance
ARC	Arcuate nucleus
BW	Body weight
cDNA	Complimentary DNA
CNS	Central nervous system
CRH	Corticotropic releasing hormone
DBB	Diagonal band of broca
DAPI	4',6- Diamindino-2-phenylindole, hydrochloride
DEPC	Diethylpyrocarbonate
DMH	Dorsomedial hypothalamus
DNA	Deoxyribo nucleic acid
DMEM	Dulbecco's modified Eagel's medium
VMH	Ventromedial hypothalamus
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GABA	Gamma aminobutyric acid
FGF	Fibroblast growth factor
GnRH	Gonadotrophin-releasing hormone
GPR54	G protein coupled receptor 54
h	Hour
Hmg	Homogenate
hCG	Human chorionic gonadotropin
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-testicular
IGF-1	Insulin-like growth factor 1
IIH	Idiopathic hypothalamic hypogonadism
ir	Immunoreactivity
IL1	Interleukin 1
im	Intramuscular
IF	Interstitial fluid
IU	International Unit
iv	Intravenous

INSL3	Insulin like factor 3
kg	Kilogram
KP	Kisspeptin
Kiss1	Kisspeptin receptor
LH	Luteinizing hormone
LIF	Lieukemia inhibing factor
MAP	Mitogen-activated protein kinase
mg	Milligram (10^{-3} gram)
min	Minute
Ml/mL	Milliliter (10^{-3} liter)
mRNA	Messenger ribonucleic acid
ME	Median eminence
MPOA	Medial preoptic area
MBH	Medial basal hypothalamus
n	Number
ng	Nano gram
NGF	Nerve growth factor
NPY	Neuropeptide Y
OVLT	Organum vasculosum of lamina terminalis
OVX	Ovarectomized
PCR	Polymerase chain reaction
PeN	Periventricular nucleus
P	Peripheral blood
PVN	Paraventricular nucleus
PIP2	Phosphatidylinositol 4,5-bisphosphate
POMC	Pro-opiomelanocortin
pM	Pico mole
PB	Phosphate buffer
P	Probability
RF-amides	Arginine-phenylalanine-amides
RPM/rpm	Revolutions per minute
RT	Reverse transcriptase
RFRP1	RFamide related peptide 1
SV	Spermatic vein blood
SEM	Standard error mean
SCF	Stem cell factor
TBS	Tris-buffered saline
T	Testosterone
TV	Testicular venous blood
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VIP	Vasoactive intestinal polypeptide
Yr	Year

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General Abstract

GENERAL ABSTRACT

Kisspeptin is an important neuropeptide involved in the regulation of reproductive axis. Its expression has been reported in reproductive axis but its complete role has not been deciphered in testicular tissue. The objective of this study was to find the expression and role of Kiss1-Kiss1r signalling in the testicular tissue of non-human primate. Three different ways have been used to find this role: firstly, to localize the Kiss1 and Kiss1r within specific cells in the testis by using single and double label immunohistochemistry and reverse transcriptase (RT-PCR). Secondly, to find any testicular effect of kisspeptin. This was done by measuring the effect of kisspeptin on testicular hormones i.e. testosterone and inhibin, from testicular fragments in incubation medium. Finally, the levels of kisspeptin were determined in testicular homogenate, testicular vein, spermatic vein and peripheral venous blood. This study was therefore based on the hypothesis that if kisspeptin is expressed in the testis then its levels should be high in testicular homogenate and it should show a concentration gradient in different testicular fluids

In the expression studies, single and double label immunohistochemistry and RT-PCR were used to localize the kisspeptin in testis. Immunohistochemistry was carried out on formaldehyde fixed tissues obtained from 6 adult rhesus monkeys by using 5 μ thick sections. Double label immunohistochemistry was used to localize the kiss1 with germ cell marker T4 and kiss1r with inhibin (a Sertoli cell marker) by using specific antibodies for them. The gene specific primers were used for carrying out the RT-PCR for kisspeptin and kisspeptin receptor. For *in vitro* study, testicular tissue fragments were obtained through testicular biopsy from five adult rhesus monkeys. The tissues were incubated in dulbecco's modified eagle's medium (DMEM) medium and treated with different kisspeptin doses for 30, 60 and 120 minutes. Kisspeptin effect was noted on testosteronem and inhibin released

in the medium. At the end of kisspeptin dose time, hCG and FSH were added to the medium for testing the competence of the Leydig and Sertoli cells in *in vitro* system.

For determining the concentration gradient of kisspeptin in testicular fluid, blood plasma was collected from testis vein, spermatic vein and peripheral vein. Kisspeptin was then measured in these fluids using enzyme linked immunoabsorbent assay (EIA).

Kisspeptin and kisspeptin receptor were found to be localized in testicular tissue of adult rhesus monkey. Immunohistochemistry showed the kisspeptin expression in spermatocytes and spermatids and kisspeptin receptor in spermatocytes and Sertoli cells. Kisspeptin expression was confirmed in the spermatids using double label Immunohistochemistry while kisspeptin receptor expression was confirmed in the Sertoli cells. In incubation study, no effect of kisspeptin was found on testosterone and inhibin release. In concentration gradient study of kisspeptin, an expected concentration gradient pattern of kisspeptin was not found in testis homogenate, testicular vein, spermatic vein and peripheral blood. Thus, our results demonstrated the expression of kisspeptin receptor in the testis. Kisspeptin has no effect on testosterone and inhibin which suggests that kisspeptin has no direct endocrine effect on testis.

We conclude that kisspeptin is present within testicular tissue but it may not have direct effects on testicular functions related to testosterone and inhibin release but it certainly has some other paracrine effects that may be more related to spermatogenesis.

General Introduction

GENERAL INTRODUCTION

Hypothalamic Pituitary Gonadal axis

The pulsatile gonadotrophin-releasing hormone (GnRH) released from hypothalamic neurons plays a pivotal role in the secretion of gonadotrophins; luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pituitary. Gonadotrophins stimulate the production of steroid hormones by the gonads which ultimately carry out gametogenesis. These series of events representing a strong interaction between brain, pituitary and gonads constitute hypothalamic pituitary gonadal axis (HPG) that ensures a successful reproduction (Plant, 2008).

Regulation of Reproduction

Reproduction is regulated at the level of GnRH system by a number of environmental and physiological factors like season, stress and effect of gonadal steroid hormones on GnRH secretion (Clarke et al., 2011). A very fundamental aspect of the HPG axis functioning is that the GnRH secretion is regulated by the sex steroids. No sex steroid receptors were found on the GnRH neurons (Herbison, 1998), therefore, various laboratories tried to find out such cells in the brain that passed on the feedback information to the GnRH cells. Discovery of the novel kisspeptin systems has completely changed our view about the regulation of GnRH secretion (George and Seminara, 2012). It seems that kisspeptin cells which operate upstream of the GnRH neurones, convey most of the steroidal effects to the GnRH neurones (Hameed et al., 2011).

Discovery and Biology of Kisspeptins

Kisspeptin peptides were encoded by the *Kiss1* gene. *Kiss1* was originally discovered as a metastasis suppressor gene in 1996. *Kiss1* is a combination of the letters ''iss'' i.e., interim laboratory nomenclature for putative suppressor sequences and the letter ''K'' that

represents the location where it was discovered (Lee et al., 1996). The *Kiss1* gene is located at chromosome 1q32 (West et. al., 1998). However, few elements at chromosome 6q16.3-q23 (Lee et. al., 1996; Miele et al., 1996; Lee and Welch, 1997; Goldberg et al., 2003; Mitchell et. al., 2007) cause upstream regulation of *Kiss1* expression (Murphy et al., 2005). *Kiss1* encodes a precursor protein of 145 amino acids that is cleaved into a 54 amino acid product (major circulating form of kisspeptin called metastin). Metastin is unstable and is further cleaved into the shorter products (kisspeptin-10, -13, -14). Metastin and other shorter fragments are collectively called kisspeptins. Different kisspeptins express similar affinity for the kisspeptin receptor (Kiss1r), which indicates that the C-terminal of the peptide is responsible for the binding and activation of the receptor (West et al., 1998; Kotani et al., 2001; Ohtaki et al., 2001). Kisspeptin belongs to the RF amide family of peptides that possess an Arg- Phe- amide sequence motif at their C-terminus (Clements et al., 2001). International committee for standardization of nomenclature has established *KISS1* and *Kiss1* terms for human and nonhuman kisspeptin genes, respectively, and the non-italicized versions of the gene nomenclature are recommended to be used for protein products of the gene (i.e., KISS1 for human and Kiss1 for other species) (http://www.informatics.jax.org/mgihome/nomen/gene.shtml). However, kisspeptin is also considered as an appropriate general term for the peptides. For the receptor, *KISS1R* and *Kiss1r* should be used for the human and non-human kisspeptin receptor genes respectively, and like the ligand, KISS1R and Kiss1r be used for the human and nonhuman receptor proteins, respectively (Gottsch et al., 2009).

Discovery and Biology of Kiss1r

In 2001, four groups working independently, recognized Kiss1 as a high-affinity peptide ligand for orphan G protein-coupled membrane receptor, GPR54 (referred to as AXOR12) (Clements et al., 2001; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Initially

described in the rats in 1999, GPR54 is now termed as"Kiss1r" because of its role as a Kiss1 receptor (reviewed in Oakley et al., 2009). *Kiss1r* gene is localized on short arm of chromosome 19 (19p13.3) and the *Kiss1r* mRNA is formed by the transcription of 5 exons of this gene (Lee et al., 1999; de Roux et al., 2003). Kiss1r is a 398 amino acid G protein-coupled receptor that is 40-45% homologous with galanin receptor. Kiss1r has a short extracellular domain that has potential N-glycosylation sites and an intracellular domain having phosphorylation activity (Lee et al., 1999). Kiss1 interaction with Kiss1r leads to a series of events including the activation of phospholipase C and cAMP transduction pathways, an accumulation of inositol-(1,4,5)-triphosphate responsible for Ca^{++} mobilization, arachidonic acid release and phosphorylation of ERK1/2 and p38 MAP kinases in CHO-K1 cell lines (Kotani et al., 2001).

Expression of kisspeptin in Vertebrate Brain

Kisspeptins are found in the genomes of many vertebrate species. Expression of Kiss1r and its consanguineous ligand, Kiss1, has been noticed in several central as well as peripheral tissues. In mice, *Kiss1* gene is expressed in the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN), arcuate nucleus (ARC), anterodorsal preoptic nucleus, medial amygdala and bed nucleus of the stria terminalis (Gottsch et al., 2004; Clarkson and Herbison, 2006). *Kiss1* gene is expressed in many areas of the rat central nervous system, including the spinal cord, medulla, pons, midbrain, thalamus, hypothalamus, amygdala, cerebral cortex, periaqueductal gray, locus coeruleus and spinal trigeminal tract. In the hypothalamus, Kiss1 peptide is highly localized in ARC, dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), medial preoptic area and paraventricular nucleus (PVN) (Dun et al., 2003; Brailoiu et al., 2005). *Kiss1* expression is sexually differentiated in some areas of the brain in the rat. In AVPV, Kiss1 cells in the adult females are 25 times more in number as compared to males (Clarkson and

Herbison, 2006; Smith et al., 2006; Kauffman et al., 2007). Kiss1 peptide is expressed in the ARC, DMH, medial preoptic area (MPOA), dorsolateral preoptic area, VMH, caudal region of the paraventricular nucleus, diagonal band of Broca (DBB), median eminence (ME) and bed nucleus of stria terminalis in the sheep (Franceschini et al., 2006; Pompolo et al., 2006). Kiss1 peptide expression has been found in the posterior and rostral ARC, organum vasculosum of lamina terminalis (OVLT) and zona interna of ME in the goat (Ohkura et al., 2009). Expression of Kiss1 peptide in horse was documented in the POA, ARC, VMH, DMH, and medial basal hypothalamus (MBH) (Decourt et al., 2008; Magee et al., 2009). In the rhesus monkey, Kiss1 neurons have been identified in the ARC, ME and MBH (Shibata et al., 2007; Ramaswamy et al., 2008). In the human brain, kisspeptin neurons have been observed in the hypothalamus, infundibular stalk, lamina terminalis, stria terminalis, infundibular nucleus, tuberal subdivision of the supraoptic nucleus and medial septum. Within the hypothalamus, kisspeptin expression has been observed in ventral periventricular nucleus, VMH, DMH, POA and PVN (Hrabovszky et al., 2008; 2010).

Many non-mammalian species also show *Kiss1* mRNA expression in the brain. In frog, *Kiss1 m*RNA is expressed in forebrain and hindbrain (Lee et al., 2009). *Kiss1* mRNA is expressed in the brain (thalamus and hypothalamic periventricular reigon) of many piscine species such as medaka, gold fish, zebra fish and sea bass (Kanda et al., 2008; Li et al., 2009; Mechaly et al., 2009; Parhar et al., 2009).

Expression of Kiss1r in Vertebrate Brain

The expression of Kiss1r is also diverse among the vertebrate species. The presence of *Kiss1r* mRNA in the rat brain was first reported in 1999 by Lee and colleagues who found its expression in pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cortex, frontal cortex, and striatum. A further detailed examination of rat forebrain revealed Kiss1r

protein expression in DBB, medial septum, medial POA, lateral POA, median preoptic nucleus, anterior hypothalamus and lateral hypothalamus (Irwig et al., 2004). *Kiss1r* m RNA expression was diagnosed in PeN and ARC areas of hypothalamus in pigs (Li et al., 2008; Tomikawa et al., 2010). In the rhesus monkey, *Kiss1r* mRNA expression was noted in the ARC, MBH and POA (Shahab et al., 2005; Shibata et al., 2007). In the human brain *KISS1R* gene expression was noted in cerebellum, cerebral cortex and brainstem (Muir et al., 2001).

Kiss1r is also expressed in the brain of many non-mammalian vertebrates including frog (hypothalamus, forebrain and hindbrain) and bullfrog (fore brain and hypothalamus) (Lee et al., 2009; Moon et al., 2009). *Kiss1r* is expressed in the brain (telencephalon including optic tectum POA, hypothalamus, midbrain, tegmentum, olfactory bulbs, optic nerves, medulla oblongata and cerebellum) of a number of piscine species such as tilapia, fathead minnow and cobia (Parhar et al., 2004; Filby et al., 2008). Two distinct transcripts of the *Kiss1r* differentiated by approximately 80 bp in length and named Ss *Kiss1r1* and Ss *Kiss1r2* are expressed in brain of goldfish (telencephalon, optic tectum, thalamus and hypothalamus) and zebrafish (mid brain, hind brain, diencephalon and telencephalon) (Biran et al., 2008; Mechaly et al., 2009).

Expression of Kisspeptin and Kiss1r in Peripheral Organs

Kisspeptin and its receptor expression are not limited to brain rather rich peripheral expression has been noted in a number of species. In the periphery, *KISS1* mRNA is highly expressed in the human placenta, testis, pancreas, liver and small intestine (Kotani et al., 2001; Muir et al., 2001).

Among the peripheral regions, expression of *Kiss1r* mRNA has been observed in the liver, intestine and pituitary of rat (Lee et al., 1999; Richard et al., 2008) and in adrenal, prostate, testis, thymus, pituitary, heart and lung of pig (Li et al., 2008). *KISS1R* is expressed in

human placenta, pituitary and pancreas. Its low levels are found in stomach, small intestine, thymus, spleen, lung, testis, kidney, and liver (Kotani et al., 2001; Bilban et al., 2004; Richard et al., 2008; Chen et al., 2011). Kiss1r is expressed in peripheral organs i.e. pituitary, heart, kidney, liver, muscle, stomach and gonads of many non-mammalian vertebrates including a number of pisceans such as tilapia, gray mullet, fathead minnow and cobia (Parhar et al., 2004; Mohamed et al., 2007; Nocillado et al., 2007; Martinez-Chavez et al., 2008). The other non-mammals in which *Kiss1r* expression has been discovered include frog (pituitary, testis, liver, heart and intestine) and bullfrog (pituitary and testis) (Lee et al., 2009; Moon et al., 2009).

Role of Kisspeptin-Kiss1r Signaling in Physiological Functions

It has been established now that kisspeptin-Kiss1r signalling plays important roles in a number of physiological functions including metastasis suppression, regulation of reproduction and metabolism (reviewed in Sempere et al., 2012; Clarke and Caraty 2013; Colledge et al., 2013). Based on initial observations, role of *Kiss1* was described as metastasis- suppressor gene in tumor biology (Lee et al., 1996; Lee and Welch, 1997a). *Kiss1* gene expression and its anti-metastasis activity has been assessed in tumour specimens of papillary thyroid carcinoma, melanoma, breast cancer and ovarian carcinoma (Lee and Welch, 1997a; 1997b; Ohtaki et al., 2001; Jiang et al., 2005). Failure of *Kiss1* gene expression was ascribed as a probable cause for metastasis and tumor development in gastric carcinoma, oesophageal squamous cell carcinoma and bladder cancer (Sanchez-Carbayo et al., 2003; Ikeguchi et al., 2004).

Role of Kisspeptin in Regulation of Reproduction

The fact that kisspeptin-Kiss1r system is a major regulator of reproduction was evidenced in 2003 by concurrent observations of two independent research groups. They inferred that loss of function mutations in *KISS1R* were the cause of the idiopathic hypothalamic

hypogonadism (IHH) and delayed pubertal maturation (de Roux et al., 2003; Seminara et al., 2003). A mutation in *KISS1R* gene was found to be responsible for IHH in a family where no involvement of GnRH receptor and GnRH gene (GnRH1) was reported (Bo-Abbas et al., 2003). To check whether these mutations alter the function of receptor, inositol phosphate production was measured in COS-7 cells expressing Kiss1r in response to kisspeptin. Inositol phosphate response of the cells with mutant receptor variants was decreased by 65% (Seminara et al., 2003). Another mutation was reported by Lanfranco and his colleagues (2005) where an insertional mutation resulted in an elongated Kiss1r protein with an elongation of 43 amino acids. This mutation resulted in delayed puberty, low testosterone and low response of LH to GnRH. In mice, targeted deletions of *Kiss1r* produced the same phenotypic abnormality of reproductive malfunction (Funes et al., 2003; Seminara et al., 2003). On the other hand, a heterozygous mutation (Arg386Pro) in *KISS1R*, in a girl caused idiopathic central precocious puberty resulting in thelarche from birth, mild increase in estrogen secretion, development of progressive secondary sexual development, accelerated growth and skeletal maturation (Teles et al., 2008). Mutation in *Kiss1* has also been shown to cause reproductive defects in male and female mice (d'Anglemont de Tassigny et al., 2007). These findings lead to the idea that *Kiss1-Kiss1r* signalling is critical for the beginning of gonadotropin secretion at puberty and to support reproduction in the adult. Absence of Kiss1 signaling in the foregoing situations is likely leading to a primary defect in the function of the hypothalamic GnRH pulse generator. On the contrary, a gain of function mutation in *KISS1R* may initiate earlier activation of GnRH pulse generator.

Role of Kisspeptin signaling in HPG axis Regulation

Kisspeptin has an important role in regulating the HPG axis. Peripheral metastin infusion causes significant increase in circulating LH, FSH and testosterone levels in the adult men

(Dhillo et al., 2005). Studies using laboratory animals (rat, mouse, and macaque) have demonstrated that kisspeptins potentenly increased LH and FSH release (Gottsch et al., 2004; Navarro et al., 2004; Plant et al., 2006). Kisspeptin-54 administration in the medial preoptic area (MPOA), rostral preoptic area (RPOA), PVN and ARC in adult male rats result in a significant increase in the plasma LH and total testosterone levels after an hour of infusion (Petterson et al., 2006). Intravenous or central administration of kisspeptin-10, results in pulsatile release of GnRH from the hypothalamus, as measured indirectly by LH, while administration of GnRH receptor antagonist (acyline) blocks kisspeptin-10 induced LH release in the agonadal rhesus monkey (Plant et al., 2006). The expression of hypothalamic *Kiss1* mRNA was observed to be increased at around the time of puberty in the male agonadal monkeys as compared to juvenile male monkeys. In ovary intact females the *Kiss1r* mRNA increased in mid pubertal monkeys as compared to juvenile (Shahab et al., 2005). Intracerebroventricular injections of kisspeptin increased the LH and FSH levels in the blood of both male and female, prepubertal and adult rats and mice (Gottsch et al., 2004; Navarro et al., 2004; Thompson et al., 2004; Navarro et al. 2005a; 2005b). Foregoing observations demonstrate that there is an increase in expression and release of hypothalamic kisspeptin which activate the kiss1r. This means that the stimulation of GnRH neurons by kisspeptin increases with the pubertal development and this signaling shifts the developmental phase from juvenile to pubertal by increasing the GnRH pulse frequency. Kisspeptins have been found to stimulate the LH secretion also in the sheep, pigs, goats, cows and horses (Abbara et al., 2013).

A good amount of data has shown that gonadotrophic system is highly sensitive to stimulation by kisspeptins and very low doses as 100 fmol to 1 pmol, injected centrally; and 0.3 nmol/kg BW, injected systemically, have been observed to significantly increase the serum LH levels in rat (Gottsch et al., 2004; Navarro et al., 2005a; Tovar et al., 2006).

It was observed that FSH release in vivo was approximately 100-folds less sensitive to the stimulation by kisspeptin than LH. A comparison of the data of kisspeptin effect on LH release and other neuropeptides and neurotransmitters like glutamate has shown that Kiss1 is the most powerful activator of gonadotropic axis. Stimulation of LH release by kisspeptin-10 is abolished in Kiss1r-null mice, in which pituitary responsiveness to GnRH is retained. This suggests that kisspeptin affects the gonadotrophic axis via Kiss1r (Messager et al., 2005).

Action of Kisspeptin on GnRH neurons

Kisspeptin has been demonstrated to act directly on GnRH neurons that have been shown to express Kiss1r in rodents and primates. Almost 90% of GnRH neurons express *Kiss1r* mRNA in both juvenile and adult mice (Han et al., 2005). However, the *Kiss1* mRNA was increased during the transformation from juvenile to adult life. With the start of puberty, kisspeptin production increases and Kiss1r signaling changes within the GnRH neurons. Administration of kisspeptin, depolarizes 90% of GnRH neurons in adult mice, and only 27% and 44%, in juvenile and prepubertal mice, respectively (Han et al., 2005). GnRH neurons co-express *Kiss1r* mRNA in rat and Kiss1 induces c-fos expression in >85% of GnRH neurons (Irwig et al., 2004; Han et al., 2005). Kisspeptin elicited GnRH secretion by rat hypothalamic explants (Thompson et al., 2004; Castellano et al., 2005). Intracerebroventricular administration of kisspeptin increased the GnRH in cerebrospinal fluid in the sheep (Messager et al., 2005). In humans, an overlapping network of GnRH and kisspeptin containing axons has been reported in circumventricular organs (Hrabovszky et al., 2008). Kisspeptin cell body number decreases during the infant-juvenile transition in the rhesus monkey. Thus a decline in pulsatile GnRH release during the infant juvenile transition may be related to a decrease in the number of kisspeptin neurons of the arcuate nucleus during this developmental transition (Ramaswamy et al.,

2013). Parenthetically, GnRH antagonists block the stimulation of the gonadotrophin by kisspeptin, in the rat, mouse and monkey (Gottsch et al., 2004; Matsui et al., 2004b; Navarro et al., 2004; Navarro et al., 2005a,b; Shahab et al., 2005). These data strongly suggest that kisspeptins control the gonadotrophic axis via hypothalamic GnRH neurons, where kisspeptins activate the GnRH secretion which leads to stimulation of LH and FSH release from the pituitary.

In the ME (internal zone and some external zone), kisspeptin and GnRH axons are in close and extensive association (Ramaswamy et al., 2008). Kisspeptin is released into the ME of the monkey in a pulsatile manner which matches exactly to the pulsatile release of GnRH in the same region (Keen et al., 2008). Kiss1 fibers were found very near to GnRH fibers in the ME, and kisspeptin causes GnRH release from rat ME in vitro (Inoue et al., 2008). In the sheep, kisspeptin neurons of the ARC project to the external zone of the ME and kisspeptin stimulates the GnRH release from ME cultured explants (Smith et al., 2011). This suggests that kisspeptin may also regulate GnRH secretion non-synaptically at the level of ME.

Factors Modulating Kisspeptin Signaling

Several factors have been shown to regulate the Kiss1 expression positively or negatively and thus modulating the HPG axis. Sex steroids positively regulate the *Kiss1* mRNA expression in the PeN and AVPV of both male and female mice through a direct effect on the Kiss1 neurons (Smith et al., 2005a; 2005b; 2006). Kiss1 neurons are present in the ARC and project to the POA where they are targeted by estrogen-positive feedback which causes metastin release into the POA in female rats (Kinoshita et al., 2005). On the other side, *Kiss1* mRNA is increased in the ARC of rats after gonadectomy which is decreased when sex steroid are administered (Navarro et al., 2004). In the ARC of ewe brain, ovarian

steroid hormone negatively regulate Kiss1 which is substantiated by the finding that ovariectomy increases Kiss1 (Pompolo et al., 2006).

Energy Balance and Kisspeptin Expression

Nutritional status is another important factor in the regulation of Kiss1 expression. Negative energy balance down-regulates the Kiss1 expression in the hypothalamus of rats which ultimately produces hypogonadotrophic state, however, normal GnRH/LH levels are restored when kisspeptin is administered to such animals (Castellano et al., 2005; Tena-Sempere, 2006). Similarly, short term fasting decreases the expression of *Kiss1* and *Kiss1r* in hypothalamus of the adult male rhesus monkey (Wahab et al., 2011)

Role of Photoperiodic Length in Kisspeptin Signaling

Photoperiodic length is an important factor which modulates the Kiss1 signaling to control reproduction in seasonal breeders. In Syrian hamsters, expression of *Kiss1* is increased in the arcuate nucleus in long days (LD) and reduced in short days (SD). A chronic administration of kisspeptin restores the testicular activity of SD hamsters (Revel et al., 2006). Kisspeptin peptide expression is higher in ARC of SD as compared to LD Siberian hamster. Acute kisspeptin injection in the Siberian hamster stimulates the LH release in both LD and SD conditions in male (Grieves et al., 2007) but only in SD conditions in female (Mason et al., 2007). Expression of *Kiss1* mRNA in the ovine ARC is increased during the breading season compared with the nonbreeding season. Kiss1 is also greater in ovarectomized (OVX) sheep and OVX sheep with the estrogen replacement in breading season (Pompolo et al., 2006; Smith et al., 2007; 2008). Interestingly, the LH/GnRH response to kisspeptin is greater during the nonbreeding season (Smith et al., 2009; Li et al., 2011). Expression of Kiss1r on GnRH neurons also increases during the nonbreeding season (Li et al., 2011).

Effects of Kisspeptin on Peripheral Tissues

Over the years, a continuous research in kisspeptinology has un-veiled many of the secrets of kisspeptin actions in different organs of a variety of species. Apart from central axis, it has now been discovered to play roles in peripheral organs as well. Kiss1r protein is expressed at notably higher levels in the neocortex of fetal adrenals during third trimester of pregnancy as compared to adult adrenals (Nakamura et al., 2007). Kisspeptin increases the aldosterone production in fetal neocortex adrenal cells. In addition, kisspeptin enhances the angiotensin II stimulated aldosterone production (Nakamura et al., 2007). Large number of pancreatic islet endocrine cells expresses high levels of Kiss1 and Kiss1r, and both of them co-localize with insulin and glucagon. This observation clearly suggests that there is a relationship between pancreatic endocrine cells and kisspeptin and it also points towards the fact that there is a local autocrine or paracrine mode of action for pancreatic islet kisspeptin (Hauge-Evans et al., 2006). Mead et al. (2007) traced a possible role of kisspeptin in the cardiovascular system of humans. Expression of *KISS1R* mRNA was noted in the aorta, coronary artery, and umbilical vein, and KISS1 and KISS1R localization was found in the atherosclerotic plaque of the coronary artery (Mead et al., 2007). It was found that kisspeptin-10, -13, and -54 may act as promising vasoconstrictors in isolated human coronary artery and umbilical vein, producing a vigorous response which is similar to the response of angiotensin-II in the coronary artery (Mead et al., 2007). Collectively, these findings indirectly refer to an unusual function of kisspeptin and its receptor in mediating the vasoconstriction, especially in blood vessels subjected to atherosclerosis. These studies highlight important associations of kisspeptins in the pathophysiology of cardiovascular disease. *Kiss1* gene is expressed in rat and human adipose tissue (Brown et al., 2008; 2009: Cockwell et al., 2012) and regulated by food intake. Food restriction increased the *Kiss1* mRNA in fat tissue in rat and high fat diet reduced *Kiss1*m RNA in

rat's fat (Brown et al., 2008). Kisspeptin administration in adult male rhesus monkeys has a stimulatory effect on adiponectin in fed and fasted conditions while kisspeptin infusion has no effect on leptin and resistin in fasting conditions. This observation points towards an adipocyte stimulatory role of kisspeptin (Wahab et al., 2010).

Role of Kisspeptin Signaling in Regulation of Reproductive Axis at the level of Gonads

It has now been well established that central Kiss1 signalling regulates the reproductive axis. Interestingly, new evidences suggest that Kiss1 may also act upon reproduction at the level of gonads. Expression of *Kiss1* and *Kiss1r* was reported in the adult rat ovary throughout the whole estrous cycle (Castellano et al., 2006). It was observed that in the ovary, levels of *Kiss1r* mRNA remain relatively constant throughout the estrous cycle, while, the levels of *Kiss1* mRNA vary irregularly, and increase on the afternoon of proestrus, directly preceding ovulation (Castellano et al., 2006). Kiss1 expression was further noted in the rat ovary and particularly found in the theca layers of growing follicles, corpora lutea, and interstitial gland (Castellano et al., 2006). These observations suggested the direct impact of locally produced ovarian kisspeptin in folliculogenesis, ovulation, and perhaps luteal function in rats. This may also apply to other animals, including humans and marmosets, where kisspeptin has been identified in the ovary (Gaytan et al., 2009). Kisspeptin from the oviduct has been suggested to be engaged in the prevention of ectopic (tubal) implantation (Gaytan et al., 2007). Kisspeptin, thus may regulate post ovulatory events in the female reproductive tract. Kisspeptin plays a role also in modulating the uterine implantation (Hiden et al., 2007).

High expression of *KISS1* and a low to moderate *KISS1R* expression was observed in human testis (Kotani et al., 2001; Ohtaki et al., 2001). However, it is not known which specific cells (Sertoli cells, Leydig cells, germ cells and peritubular cells) in the testis

express Kiss1 and Kiss1r. The foregoing studies did not use the techniques which could specify the morphological and histological details associated with kisspeptin and its receptor in the testis. These studies mainly used PCR based techniques only. Therefore, the role of kisspeptin in the testis as a paracrine modulator could not be identified. Expression of *Kiss1 and Kiss1r* in specific cells of testis may decipher the role of Kiss-Kiss1r signaling in the paracrinology of testicular functions.

Morphology of Testis

The testes are primary reproductive organs in the male. The human testis is enclosed in a connective tissue capsule, the tunica albuginea within which smooth muscle fibers are found (Kerr et al., 2006). The mammalian testis is considered to be divided into two compartments, tubular compartment consisting of seminiferous tubules and an interstitial compartment present between the tubules (Kerr et al., 2006; Weinbauer et al., 2010). Tubular compartment contains the germ cells and two different types of somatic cells, the peritubular cells and the Sertoli cells. The testis is divided into lobules by connective tissue septa which contain convoluted seminiferous tubules (Weinbauer et al., 2010). Interstitial compartment contains, in addition to blood vessels, Leydig cells, macrophages and other minor components (Sharpe, 1984). The seminiferous tubules constitute 90% of the testicular tissue. Continuous cell multiplication involved in spermatogenesis requires nutrition and energy in the tubule (Setchell, 1978). In many mammals, an interstitial fluid (IF) surrounds the seminiferous tubules and fills the interstitial spaces. This fluid transports all hormones and nutrients from the bloodstream and is formed by filtration from the capillaries (Fawcett et al., 1973; Clarke, 1976). The human seminiferous tubules are covered by a basal membrane, (a layer of collagen) and the layer of peritubular cells. Several factors are expressed by peritubular cells such as panactin, desmin, gelsolin, smooth muscle myosin and actin that are involved in cellular contractility (Holstein et al.,

1996). These cells have an important role of transporting mature sperms towards the exit of seminiferous tubules by contractions (Weinbaur et al., 2010). Peritubular contractility is mediated by endothelin and this effect is modulated by the relaxant peptide adrenomedullin produced by Sertoli cells (Romano et al., 2005).

Sertoli cells are somatic cells, named after Enrico Sertoli an Italian physiologist. They are located on the basal membrane and extend to the lumen of the tubules. Morphology of Sertoli cell is connected to their function. They are responsible for determining the final testicular volume and sperm production in the adult (Weinbaur et al., 2010). Importance of the foregoing is demonstrated by the evidence that decrease of Sertoli cell numbers produced by an antimitotic substance leads to a reduction of testicular volume and sperm production (Weinbaur et al., 2010). Sertoli cells build tight junctions between each other, thus forming the blood testis-barrier. Blood-testis-barrier divides the seminiferous epithelium into basal and adluminal reigons which are different in their anatomy and function. Germ cells are transported from basal to adluminal area during their division through dissolving of blood testis barrier. Earlier stages of germ cells are located within the basal reigon and the maturing germ cells are located in the adluminal reigon (Kerr et al., 2006).

Various germ cells are arranged in typical cellular associations within the seminiferous tubules known as spermatogenic stages. In humans, spermatogonia are present at the base of the seminiferous epithelium and are of two types i.e. A and B. From a cytological point of view, type A spermatogonia can be further divided into two types, the Ad (dark) and the Ap (pale). The Ad spermatogonia have been found to have no proliferating activity under normal circumstances (Russel et al., 1990), while, the Ap spermatogonia divide into two B spermatogonia (Ehmcke and Schlatt, 2006): B spermatogonia are converted into the preleptotene spermatocytes (primary spermatocytes) directly without any meiotic division

(Alastalo et al., 1998). Spermatocytes go through different phases of the meiotic division leading to secondary spermatocytes (with haploid chromosomal set in duplicate form) and haploid spermatids. Spermatids are mitotically inactive cells, which, by condensation, structural rearrangement, formation of a flagellum and the expulsion of a large part of cytoplasm are differentiated into sperm. The main event during the maturation phase of the spermatids is the expulsion of the rest of the cytoplasm as the residual body. Residual bodies are phagocytosed by Sertoli cells and have a regulatory role. Elongated spermatids and their residual bodies influence the secretory function of Sertoli cells (production of tubular fluid, inhibin, androgen-binding protein and interleukin-1 and 6). The release of sperm into the tubular lumen is called spermiation and it is influenced by hormonal modifications (Kerr et al., 2006).

Functions of the Testis

Testis performs two main functions; spermatogenesis, production of mature sperm from spermatogonia and steroidogenesis production of testosterone. Spermatogenesis takes place in the tubular compartment and testosterone production in the Leydig cells.

Spermatogenesis involves the conversion of an undifferentiated stem cell (type A spermatogonium) into a highly differentiated immature spermatozoan. These germ cells are in close association with Sertoli cells. Sertoli cells provide most of the nutritional and physical support for the developing germ cells (Sharpe, 1983). Spermatozoan is produced from a type A spermatogonium through successive stages and in each stage different germ cell types are associated (Clermont, 1972). These stages follow each other, so that along the length of an individual seminiferous tubule in rat, stage I will be followed by stage II and preceded by stage XIV. This spatial distribution is termed as the spermatogenic wave (Kerr and de Kretser, 1981). Specific stage has its own requirements of Sertoli cells secretions for the developing germ cell (Waites and Gladwell, 1982).

Testicular steroidogenesis involves the production of androgens within the interstitial compartment (Leydig cells) of the testis (Kerr et al., 2006). The process is carried out under the control of the pulsatile release of the LH acting through the LH receptor on the surface of the Leydig cells (Dufau, 1996). Testosterone is the most biologically important androgen synthesized in the testis. Testosterone synthesized by the Leydig cells can be secreted into the circulatory system or it can be metabolized further to its highly active metabolite, dihydrotestosterone in the Leydig cells. Testosterone is synthesized by a sequence of enzymatic reactions that convert the substrate cholesterol, initially into the pregnenolone, and then through a number of steroid intermediates to testosterone (Hall, 1994).

Regulation of Testicular Functions

Functions of testis are regulated by a classical pituitary input or by a local regulation.

Central Regulation of Testicular Functions

Hypothalamus and pituitary have been demonstrated to be involved in regulating both the functions of testis. In turn, testicular hormones have also been known to carry out the negative feedback regulation of the HPG axis (Leutjens et al., 2005). Testosterone inhibits the secretion of LH and FSH and inhibin inhibits FSH (Boepple et al., 2008). Either LH or FSH alone is capable of initiating and maintaining spermatogenesis only qualitatively while quantitative maintenance of spermatogenesis requires both LH and FSH (Weinbauer et al., 2004). It was found that in patients with activating mutations of the LH receptor or testosterone producing Leydig cell tumors, complete spermatogenesis occurs. This suggests that high local levels of testosterone induce sperm production. Treatment of such patients with human chorionic gonadotropin (hCG), which contains high LH activity, maintained spermatogenesis. On the other hand, patients having a defective FSH β subunit, had azoospermia (Lindstedt et al., 1998; Phillip et al., 1998). In men, in whom,

gonadotropin is suppressed; either FSH or LH maintained spermatogenesis (Matthiesson et al. 2006). FSH can also stimulate Leydig cell numbers in immature primate testis. These stimulatory effects have been observed in the absence of endogenous LH (Haywood et al., 2003) and are mediated via the FSH receptor. Gonadotropins are also involved in activating the Sertoli cell proliferation (Schlatt et al., 1995; Plant et al., 2005).

Regulation of Testicular Functions by Local Testicular Factors

Apart from the central endocrine regulation, it has been found that complex machinery of cells within the testis is also involved in controlling the testicular functions. The local factors may be paracrine (act on neighbouring cells by diffusion between them) and autocrine (factors which act on the same cells from which they are released). The local interactions of testicular cell are also referred to as paracrinology (Weinbauer and Wessels, 1999). Local factors are considered as regulators of pituitary hormone action and intracellular communication (Weinbaur et al., 2001). Local interactions also involve the interplay between the different testicular compartments (Weinbauer et al., 2010). This communication exits between peritubular cells and Sertoli cells, between Sertoli cells and germ cells and between germ cells themselves (Skinner et al., 1991).

Sertoli cells establish numerous morphological and structural connections with germ cells and control the spermatogenesis in all aspects. Moreover, there is a stage specific organization of Sertoli cells in epithelium (Jegou et al., 1992). Blood testis barrier formed by the Sertoli cells establishes a special environment for the meiotic process and sperm development as well as it is the physical isolation of haploid germ cells to prevent recognition by the immune system (Weinbauer et al., 2010). Sertoli cells keep interaction with germ cells through the secretion of many factors and participate in the local regulation of spermatogenesis. These factors include growth factors, cytokines, transferrin, connexion-43, plasminogen activator and many other molecules. The growth factors

secreted by Sertoli cell include transforming growth factor (TGF)α and β, insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), inhibin, activin, nerve growth factor (NGF), and epidermal growth factor (EGF). Generally, TGFα and IGF-I have a stimulatory action in the testis, while TGFβ acts as an inhibitor. FGF and IGF have been found on Sertoli cells and germ cells. IGF-I concentrations are positively related to the number of pachytene spermatocytes. In men, IGF-I shows the highest expression in pachytene spermatocytes and stimulates DNA synthesis in mitotic germ cells (Skinner et al., 1991). EGf and TGF have been identified to be involved in the survival and proliferation of type A spermatogonia and FGF in mitosis of spermatogonia and Sertoli cell-germ cell interactions. NGF has important role in the structural organization of the human seminiferous tubules and is an important regulator of meiotic division in the rat (Hneji et al., 1991). Inhibins have been detected in primate Leydig cells and Sertoli cells (Vliegen et al., 1993). Activins are considered to stimulate spermatogonial proliferation whereas inhibins exert inhibitory actions. It has been found that serum concentrations of inhibin are related to spermatogenic activity, testis size and sperm production (Meachem et al. 2001; Boepple et al. 2008). Connexin 43, a transmembrane protein, is involved in Sertoli cell maturation and division (Brehm et al., 2007; Sridharan et al., 2007). Immune factors may also play a more direct role during testicular steroidogenesis and gametogenesis (Hedger, 2002; Fijak and Meinhardt, 2006). These factors involve leucocyte, macrophage and mast cell products. Sertoli cells also secret some cytokines such as interferon, tumor necrosis factor (TNF), interleukins, leukemia inhibing factor (LIF), and stem cell factor (SCF). TNF and LIF are thought to play a role in Sertoli cell-germ cell interactions (Benahmed, 1997) and LIF has been implied in the autocrine control of Sertoli cell proliferation (Hara et al., 1998). Interleukin 1 (IL1) has a role in DNA synthesis in spermatogonia in rat testis (Cohen and Pollard, 1998). SCF is synthesized and secreted by

Sertoli cells and its receptor is expressed on spermatogonial surfaces. SCF and its receptor (c-kit) are essential local factors that monitor germ cell migration during spermatogonial differentiation in the adult testis (Loveland and Schlatt, 1998). Secretion of plasminogen activator by the Sertoli cells is stage specific and increases 10- to 30-fold at stages VII and VIII of the cycle than at other stages of the spermatogenesis (Sharpe, 1983). Transferrin is secreted preferentially at stages IX-XII of the cycle. Transferrin is important for supplying iron to germ cells in the adluminal compartment of the Sertoli cell (Skinner and Griswold, 1980).

Testosterone, a classic endocrine factor, has also been demonstrated to act as local regulator of spermatogenesis. In rodent, selective elimination of Leydig cells, interruption of testicular testosterone transport and specific Sertoli cell androgen receptor knockout models caused alterations of germ cell maturation (Takaimya et al., 1998). Peritubular cells express the androgen receptor in the non-human primate and testosterone induces the formation of smooth muscle actin in the peritubular cells during prepubertal testicular maturation (Schlatt et al. 1993). FSH receptors are found only on Sertoli cells suggesting that FSH influence testosterone action indirectly through Sertoli cell factors (Schlatt et al., 1993). Recombinant FSH stimulates testosterone production by Leydig cells in men through a Sertoli cell secreted non steroid factor which amplifies the LH response (Levalle et al. 1998) and in patients with selective FSH deficiency by paracrine mechanism on Sertoli cells (Lofrano-Porto et al., 2007).

It is now established that Sertoli cells support germ cells in every possible way, on the other hand, there are also evidences supporting the fact that germ cell might control the activity of Sertoli cells. Administration of specific testicular toxins for the elimination of specific germ cells, stimulated changes in the secretion of inhibin from Sertoli cells (Jegou,

1993; Sharpe, 1994). Sertoli cells have variable requirements for metabolic substances depending on the phase of spermatogenic cycle and are under the local control of germ cells (Franca et al., 1998). Pachytene spermatocytes and early stage round spermatids, have been shown to influence Sertoli cell function. Germinal cell conditioned medium stimulates the phosphorylation of specific proteins and increase in transferrin gene expression activity in Sertoli cells. NGF gene expression has been observed in spermatocytes and early spermatids (Skinner et al., 1991). NGF has been shown to increase the viability of Sertoli cells in culture and to increase the levels of androgen binding protein (Lonnerberg et al., 1992; Parvinen et al., 1992; Chen et al., 1997). Germ cells secrete such factors which act on other germ cells in seminiferous epithelium and in this way such germ-germ cells interaction is also an important part of testicular paracrinology. The OCT-4 transcription factor is expressed by type A spermatogonia of the adult mouse testes and it is involved in enhancing the activity of spermatogonia (Pesce et al., 1998). Neurogenin 3(Ngn3) is expressed by the earliest spermatogonia (Yoshida et al., 2004). GFRa (receptor of glial cell line-derived neurotrophic factor) is expressed by early spermatogonia and has a role in stem cell self-renewal. SALL4 is a zinc finger transcription factor and a spermatogonial marker (Elling et al. 2006). In vitro, SALL4 stimulates embryonic stem cell proliferation (Phillips et al., 2010)

Kisspeptin: A Neuropeptide acting as Local Testicular factor

It is interesting to mention that many neuropeptides are expressed in the testis including GHRH, CRH, ACTH and GnRH (Weinbaur et al., 2010). Gonadotropin inhibitory hormone (GnIH) has been recently added to the list of neuropeptides and factors which are expressed by germ cells of testis. GnIH (RFRP) peptide expression was described in spermatids in hamster testis. RFRP receptor (GPR147) was found in late pachytene spermatocytes (Zhao et al., 2010). GnIH expression has also been determined in the germ

cells of rhesus monkey (Bentley et al., 2010). Kiss1 expression in human testis may provide a new paracrine player (Kotani et al., 2001). Peripheral kisspeptin administration in acyline clamped pituitary of rhesus monkey stimulated hCG dependent testosterone release (Irfan et al., 2008). Moreover, in adult male monkeys, continuous intravenous infusion of kisspeptin invariably exaggerated plasma testosterone concentrations for a given LH stimulus (Ramaswamy et al., 2007). These factors point towards an involvement of Kiss1-Kiss1r signaling in regulating testicular functions. Recently the presence of KISS1 and its receptor have been demonstrated in mature human spermatozoa and small, but significant changes in sperm motility were induced by KISS1 that was reduced in the presence of the receptor antagonist p234 (Pinto et al., 2011). Thus, KISS1, acting via KISS1R could activate the signal transduction pathways to modulate sperm movement in humans (Pinto et al., 2011). Foregoing evidence strongly suggests a local action of Kiss1 in the primate testis. However, there is still a lack of information about precise location of action of Kiss1 in the testis. The previous studies utilized only RNA expression techniques like reverse transcriptase PCR (RT-PCR) and in situ hybridization which do not highlight the morphology of the testis. Characterization of specific cells within the testis and specifically of germ cell types may have an important role in unveiling the kisspeptin as a direct local regulator of testis. The present study was therefore, designed to characterize the expression of Kiss1 and Kiss1r in the testis of non-human primate using rhesus monkey as a model and also to evaluate the functional significance of this expression with respect to the testosterone and inhibin secretion. Parenthetically, it was also sought to measure the levels of kisspeptin in various testicular circulation fluids. Objectives of the present study were, therefore,

Objectives of the Present Study

1: To study the expression of Kiss1 peptide and Kiss1r protein in the testis of adult male rhesus monkey. This was done specifically by:

a. Examining the expression of Kiss1 and Kiss1r mRNA analysis in adult male rhesus monkey testis by using reverse transcriptase PCR, and

b. Localizing the Kiss1 and Kiss1r protein on testicular cells using double label immunohistochemistry.

2. To evaluate the functional significance of Kiss1 and Kiss1r expression in the testis of adult rhesus monkey. This was done by challenging different kisspeptin doses to a culture of testicular tissue of adult rhesus monkey and assessing testosterone and inhibin responses in vitro.

3. To verify the possibility of release of kisspeptin from the testis tissue because of its local production, a comparison of kisspeptin levels was done in rhesus monkey testis homogenate preparation, testicular vein, and spermatic vein.

General Materials and Methods

GENERAL MATERIALS AND METHODS

Animals

A total number of eleven adult intact male rhesus monkeys (*Macaca mulatta*), 7-9 years old, weighing 8.3 -14.7 kg were used in the studies described in this thesis. Their testicular volumes were 19.1-61.4 ml. The animals were housed in individual cages and maintained under semi-controlled environmental conditions with temperature (25±3°C) and light (06:00-18:00) in the Primate Facility of the Qauid-i-Azam University, Islamabad. Standard monkey diet supplemented with fresh fruits (09:00-09:30 h), boiled eggs (11:00 h) and bread (13:00-13:30 h) was provided daily. Water was available *ad libitum*. All animal experiments were approved in advance by the Departmental Committee for Care and Use of Laboratory animals.

A formal power analysis was not carried out to determine the animal sample size to be statistically correct. The selection of sample size was rather based on literature studies. Moreover, as primates are expensive animals so their sample size was taken small in an experiment.

Fluorescence Immunohistochemistry

Tissue Collection

For immunohistochemical studies, testes were obtained from 6 adult intact male rhesus monkeys weighing 6.8-14.7 kg and their mean testicular volume was 45 ml ± 1. The monkeys were sedated with ketamine hydrochloride (Ketler, Astarapin, Germany) given at a dose of 10 mg/kg body weight (BW) intramuscularly and then euthanized with ketamine hydrochloride 25-30 mg/kg BW intravenous injection. These animals were euthanized for taking hypothalami to be used in another study (Wahab et al., 2010). After removing the

tunica albugenia, about 100 mg testicular tissues were obtained from anterior pole of left testis of each animal. The testicular tissues were immediately transferred to Sera fixative. The experiments were carried out during September 2009.

Tissue Fixation and Processing

For immunohistochemistry, testicular tissues were immersion-fixed in formaldehyde-alcohol-acetic acid fixative (60 ml absolute alcohol (Sigma-Aldrich GmbH, Steinheim, Germany), 30 ml of formaldehyde (Merck, Darmstadt, Germany) and 10 ml of glacial acetic acid (Merck) for 4-5 hours. The tissues were then passed through graded alcohols and placed in cedar wood oil (Serva Electophoresis, GmBH, Heidelberg, Germany) until they appeared clear. Tissues were then washed twice (10 min) with benzol, placed in benzol+paraplast (1:1) (Fluka AG, Buchs, Switzerland) for 20 min and then washed 3 times (3 x 12 h) in Paraplast (Fluka AG, Buchs, Switzerland) at 60 °C to keep it in liquid state. Finally, the tissues were embedded in paraffin and sectioned at 5 µm on a Shandon Finesse 325 microtome (Thermo Electron Cooperation, Waltham, MA, USA).

Immunohistochemistry Procedure

Paraffin embedded 5 µm thick sections were de-waxed in xylene (Merck, Darmstadt, Germany), rehydrated through descending grades of alcohol and rinsed in distilled water. The sections were then washed in 0.1 M Tris-buffered saline (TBS) (pH 7.4), mounted on superfrost slides (Santa Cruz Biotechnology, Heidelberg, Germany) and dried over-night. Antigen retrieval was then performed using citrate buffer (pH 6.0) and heating in a microwave oven (2x3 min). The slides were then washed in TBS (3x10 min) and incubated in following blocking solution (10% normal serum of the specie in which secondary antibody was raised + 0.3 % Triton X-100 (Merck, Darmstadt, Germany) in TBS) for 2 h at room temperature. In the case of double label, immunohistochemistry procedures for both the antigens were run separately till the secondary antibody incubation. Sections were

incubated in primary antibodies (and in a cocktail of primary antibodies in case of double fluorescence) prepared in TBS containing 10% normal goat serum and 0.3% Triton X-100 at 4 °C for 72 h. The antibodies used are shown in table 1. The sections were then washed in TBS and incubated with relevant secondary antibodies in phosphate buffer (PB) for 2 h and counterstained (3 min) with 0.3% Sudan black B (dissolved in 70% ethyl alcohol). The sections were then mounted with a preparation of an anti-fade fluorescent medium (DAKO, Carpentaria, CA, USA) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; nucleic acid stain, Molecular Probes, Eugene, Oregon, USA). Sections were then coversliped and kept at 4 °C until examined.

Microscopy

Slides were viewed under 20x and 40x objective lenses on a Zeiss AX10 microscope with fluoresence lamps (Carl Zeiss, Inc, GMBH, Jena, Germany). Fluorescence images of single or double labeling were created using Zeiss image analysis software by using fluorescence filters: Alexa fluor 488, Alexa fluor 546 and texas red 546. Single label fluorescence images for kisspeptin and inhibin, and kisspeptin and T4 were merged to get double labeled images.

Table 1. Primary and Secondary Antibodies used in Immunohistochemistry

Tissue	Peptide	Primary Antibody	Dilution	Reference of specificity	Source
Testis	Kiss1	Sheep anti human(GQ2)	1:120,000	Ramaswamy et al. (2009).	Prof Dr. Stephen Bloom (Imperial College, London, UK).
Testis	Kiss1r	Rabbit anti-AXOR12	1:1000	Bilban et al. (2004).	Phoenix Pharmaceuticals, Brisbane, Australia
Sertoli cell	Inhibin	Mouse monoclonal anti human inhibin alpha(R1)	1:100	Groome et al. (1990).	Serotec, Melbourne. Australia
Leydig cell	17β-HSD3	Goat anti-17β-HSD3(c-14)	1:100	Nanjappa et al. (2012).	Santa Cruz Biotechnologies, Melbourne, Australia
Sperm	TPX1(now called CRISP2)	Rabbit anti T4	1:200	O'Bryan et al. (2001).	Dr. Moira O'Bryan (Monash Institute of Reproduction and Development Melbourne, Australia)

Testicular Tissues Biopsy

Testis tissues were collected from five intact adult rhesus monkeys for in vitro experimentation. The animals were anesthesized with intramuscular injection of 10 mg/kg BW ketamine hydrochloride. An incision was given in the anterior side of scrotum with a sharp scalpel blade. The testes were exteriorized from the scrotum. Firstly, 1 ml of testicular vein blood (TV) and spermatic vein blood (SV) were taken from left testis with the help of a heparinized 1 ml/cc 27 gauge syringe. Secondly, testis biopsy was carried out on the same tissue for RT-PCR, testis homogenate preparation and testiscular incubations. For testicular biopsy, a 2-3 cm incision was given in the tunica vaginalis, at the proximal side of the testis, avoiding veins, so that the testis parenchyma was exposed. A total of 700 mg testicular tissue was taken carefully from each animal. For RT-PCR and testis homogenate preparation, 100 mg tissue per study was taken from each monkey testicular tissue, weighed and immediately preserved in liquid nitrogen and then saved at -70 °C. For testis incubation studies, five fragments of testis tissue (each 100 mg) were collected, weighed and kept in ice chilled incubation medium (Dulbecco's modified eagle's medium (DMEM)) and fetal calf serum (FCS). Afterwards, tunica vaginalis, peritonium and skin each were sutured separately. A preparation of penecillin and streptomycin was sprinkled on the wound to avoid infection. All the instruments were autoclaved before use. A single shot of long-acting antibiotic was given after surgery. Stitches were removed after 2 weeks.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Tissue Collection

For the measurement of mRNA, by RT-PCR, approximately 100 mg of testicular tissues were collected from biopsied tissues of each monkey under sterile surgical conditions.

These tissues were immediately frozen in liquid nitrogen and kept at -70 °C until used for RNA extraction.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from testicular tissue using the TRIzol reagent (Invitrogen Life Sciences, Carlsbad, CA, USA) and reverse transcribed with a First Strand cDNA Synthesis kit (Fermentas, Osaka, Japan). Kiss1 and kiss1r primers were designed to target a segment comprised within the cloned cDNA. Primers sequences were selected as described by Wahab et al. (2010). The primer sequences were synthesized by Fermentas and ThermoFisher Scientific (Osaka, Japan) and were as follows:-

Kiss1r: forward - 5'-CTCGCTGGTCATCTACGTCA-3',

reverse -5' -CGAACTTGCACATGAAATCG-3'

Kiss1: forward -5'-CTGGAATCCCTGGACCTCTC-3'

reverse 5'-TTGTAGTTCGGCAGGTCCTT-3'.

Amplification of cDNA was carried out in a reaction mixture of 50 µl. The mixture consisted of 3µl of cDNA, 0.7 µl Taq polymerase, 35 µl RNAse free water, 2.5 µl of each forward and reverse primer, 5 µl reaction buffer and 1.3 µl dNTP. PCR conditions were: 10 min at 95°C, 40 cycles of 30 seconds (s) at 95°C, 1 min at 60°C, 30 s at 72 °C using a thermocycler (Whatman Biometra, GmBH, Goettingen, Germany). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as an internal control.

Pharmacological and General Reagents

The following pharmacological reagents were used in this study.

1. Ketamine hydrochloride (Ketler, Astarapin, Germany)
2. Heparin (Rotex medica, Trittau, Germany)
3. Human kisspeptin-10 (KP10; amino acid 112-121; Calbiochem, La Jolla, CA, USA)
4. TRIzol® (Invitrogen Life Sciences, Carlsbad, CA, USA)

5. Human chorionic gonadotropin (hCG; IVF-C, Iksan-si, Jeonbuk-do, Korea)

6. Normal saline (0.9% NaCl; A-Z pharmaceuticals, Kasur, Pakistan).

7. Follitropin beta (Follicular stimulating hormone; Puregon, Organon, Oss, Netherlands)

8. DMEM (Invitrogen, Gibco, N.Y, USA)

9. FBS (Fetal Bovine Serum, Hyclone, Cramlington, UK)

In Vitro Studies

Testicular Tissue Incubation

Analysis of the direct effect of kisspeptin on testosterone and inhibin secretion was carried out by using the testicular slice incubation method which was done as previously described by Roulet et al. (2006) with slight modifications. Testicular tissues were biopsied from adult rhesus monkeys. The tissues were de-capsulated, weighed and cut into five fragments of approximately 100 mg which were immediately transferred to fresh ice cold Dulbecco's modified Eagle's medium (DMEM) with PH 7.4. Testicular slices were incubated for 1 h in 2 ml DMEM containing 1.2 g/l sodium bicarbonate, glucose (1 mg/ml), 50 IU/ml penicillin, 50 µg/ml streptomycin and 10% FBS in 5 ml culture tubes under 5% CO_2 and 95% air at 32 °C in a CO_2 incubator. After incubation for 1 h, the media was replaced with medium containing kisspeptin at five different dose rates (no dose, 1pm, 10pm, 100pm, 1000pm). Aliquots of 100 µl were then taken from each tube after 30, 60 and 120 min of incubation and stored at -20 °C until assay. Then, all media was removed and replaced with media containing 10IU HCG. HCG incubation was used to evaluate the viability of the Leydig cells to secrete testosterone in the culture conditions. 100 µl aliquots of the medium were taken after 3 h. Again all the media was replaced with medium containing 100 ng/ml FSH and 100 µl aliquots were taken after 2 h. FSH incubation was used to measure the viability of Sertoli cells to secrete inhibin.

Testicular Homogenate Preparation

The testis homogenate preparation was done as previously described by Laurenzana et al. (2002). For the preparation of testis homogenate 100 mg of testis was taken from biopsied fragments of testis and immediately frozen in liquid nitrogen. For homogenate preparation, the testis were thawed and homogenized in 100 µl of 50 mM phosphate buffer (pH 7.4) using a pestle morter. Aliquots of the homogenates were stored at −80 °C until kisspeptin assay.

Collection of Testicular Vein Blood, Spermatic Vein Blood and Peripheral Blood from Adult Rhesus Monkey

The method for testicular venous blood, spermatic vein blood and peripheral blood collection was adopted as described by Waites et al. (1974). Five adult intact rhesus monkeys were used in the study. Each testis was exposed through a 2-3 cm incision in the anterior aspect of the scrotum under ketamine anesthesia. Testis was removed from the scrotum. For the collection of testicular venous blood from the testis, heparin coated insulin syringe was inserted in the testicular vein on the surface of the testis, and 1ml blood was slowly collected from the testicular vein (Figure 1). Testicular vein was differentiated from the artery on the basis that veins run towards the proximal part of testis and form branches there while artery run around distal part of testis. Blood samples were also collected from one of the spermatic veins at the proximal end of the spermatic cord into heparinized tubes (Figure 1). Blood from a peripheral vein (saphenous vein) was also collected.

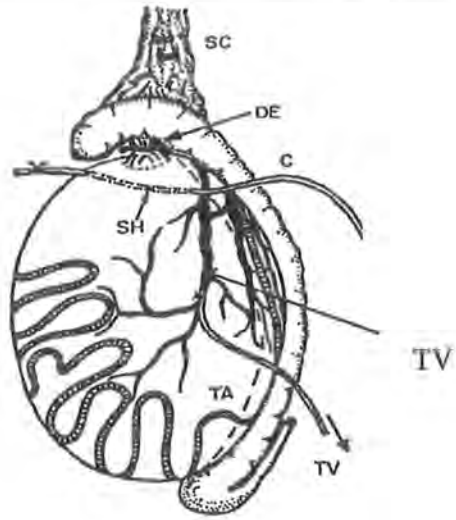

Figure 1. Diagrammatic representation of testicular vasculature of a monkey testis. Blood was collected from one of the spermatic veins of spermatic cord (SC) and from a testicular vein (TV).

Hormone Assays

Plasma testosterone, inhibin, and kisspeptin concentrations were determined by using specific enzyme linked immunoabsorbent assays. Testosterone, inhibin and kisspeptin kits were obtained from the following companies, respectively, Amgenix Microlisa™ (San Jose, CA, USA), Beckman Coulter (Brea, CA, USA) and Phoenix pharmaceuticals (Burlingam, CA, USA). The assays were performed according to the manufacturer's instructions.

The sensitivity of the testosterone assay was 0.05 ng/ml and intra- and inter-assay coefficients of variation were both < 10%. Sensitivity of the inhibin assay was 2.6 pg/ml and the intra- and inter-assay coefficient of variation was < 6%. The sensitivity of the Kiss1 (68-121) amide/metastin (1-54) amide (human) assay was 0.13 ng/ml and intra assay variation was < 10% and inter assay variation was < 15%. The assay shows 100% cross

reactivity with metastin (1-54) amide /Kiss1 (68-121) amide (human), metastin (45-54) amide/Kiss1 (112-121) amide (human) and RFRP1 (human).

Testosterone EIA

Principle of the Assay

The testosterone EIA is based on the principle of competitive binding between testosterone in the test specimen and testosterone-HRP conjugate for a constant amount of rabbit anti testosterone. Goat anti rabbit IgG coated wells are incubated with 10 µl of testosterone standards, controls, samples, 100 µl testosterone HRP conjugate reagent and 50 µl rabbit anti testosterone reagent at 37°C for 90 minutes. During the incubation, a fixed amount of HRP labeled testosterone competes with the endogenous testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific testosterone antibody. Thus the amount of testosterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of the testosterone in the specimen increases. Unbound testosterone peroxidase conjugate is then removed and the wells are washed. Next, a solution of the tetramethylbenzidine (TMB) reagent is then added and incubated at room temperature for 20 minutes, resulting in the development of blue colour. The colour development is stopped with the addition of 1N HCl and the absorbance is measured spectrophotometricaly (Microplate Reader, BIO RAD, Tokyo, Japan) at 450 nm. The intensity of the colour formed is proportional to the amount of unlabeled testosterone in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The testosterone concentration of the specimens and controls run concurrently with the standards thus can be calculated from the standard curve.

Assay Procedure

Coated wells were secured in the holder and 10 µl of standards, specimens and controls were added into appropriate wells. 100 µl of testosterone-HRP conjugate reagent and 50 µl of rabbit anti testosterone reagent were then dispensed into each well. They were mixed and incubated at 37°C for 90 minutes. The microwells were then rinsed and flicked 5 times with distilled water. Subsequently, 100 µl of TMB was dispensed into each well, gently mixed for 10 seconds and incubated at room temperature (18-25°C) for 20 minutes. The reaction was stopped by adding 100 µl of stop solution to each well and gently mixed for 30 seconds till all the blue color changed to yellow completely. Absorbance was read at 450 nm with a microplate reader within 15 minutes.

Inhibin ELISA

Principle of the Assay

The inhibin B ELISA is an enzymatically amplified three-step "sandwich" assay. In the assay, calibrators, controls and samples are incubated in microtitration wells which have been coated with anti-activin B antibody. After incubation and washing, the wells are incubated with biotinylated anti-inhibin α-subunit detection antibody. After a second incubation and washing step, the wells are incubated with streptavidin labeled with the enzyme horseradish peroxidase (HRP). After a third incubation and washing step, the wells are incubated with the substrate TMB and an acidic stop solution is added. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as primary reference filter. The absorbance measured is directly proportional to the concentration of inhibin B in the samples. A set of inhibin B calibrators is used to plot a calibration curve of absorbance versus inhibin concentration. The inhibin concentrations in the samples can then be calculated from this calibration curve.

Assay Procedure

Following reagents were first prepared as given:

1. Wash Solution: 1 part wash concentrate B was diluted with 24 parts of deionized water.

2. Inhibin B antibody-biotin conjugate: The inhibin B antibody-biotin conjugate concentrate was diluted (10–30 minutes prior to use) at a ratio of 1 part into fifty parts of inhibin B biotin conjugate diluent, according to the number of wells used. For an entire plate, 220 µL of the concentrate was pipetted into 11 mL of the inhibin B biotin conjugate diluent.

All samples and reagents were allowed to reach the room temperature (~25 °C). The reagents were mixed thoroughly by gentle inversion before use. Calibrators and controls were assayed in duplicate. The microtitration strips to be used were marked. Fifty µL of the calibrators, controls and samples were pipetted in the appropriate wells. Then, 50 µL of the inhibin B assay buffer was added to each well. The wells were then incubated for two hours at room temperature (~25 °C), with shaking at 600–800 rpm on an orbital microplate shaker.

During the last 10-30 minutes of incubation, the inhibin B antibody-biotin conjugate solution was prepared by diluting the inhibin B biotin conjugate concentrate in inhibin B biotin conjugate diluent. Each well was washed five times with the wash solution manually. Plate was blot and dried by inverting plate on absorbent material. Inhibin B antibody-biotin conjugate solution of 100 µL was then added to each well. The wells were then incubated with shaking at 600–800 rpm on an orbital microplate shaker, for 60 minutes at room temperature (~25 °C). Again, each well was washed five times with the wash solution and blot dried by inverting plate on absorbent material. 100 µL of the inhibin B streptavidin-enzyme conjugate solution was then added to each well using a precision pipette. Next, the plate was incubated with shaking at 600-800 rpm on an orbital microplate

shaker, for 30 minutes at room temperature (~25 °C). Each well was washed five times with the wash solution using an automatic microplate washer and blot dried by inverting plate on absorbent material. 100 µL of the TMB chromogen solution was added to each well followed by addition of 100 µL of the stopping solution to each well. The absorbance of the solution in the wells was then read within 30 minutes, using a microplate reader set at 450 nm.

Kisspeptin EIA

Principle of Assay

The immunoplate is pre-coated with secondary antibody and the nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (kisspeptin antibody) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. The intensity of the yellow colour is directly proportional to the amount of biotinylated peptide- SA-HRP complex but inversely proportional to the amount of the kisspeptin in the standard solutions or samples. This is due to the competitive binding of the biotinylated peptide with the standard peptide or samples to the kisspeptin antibody. A standard curve of known standards vs. absorbance can be established accordingly. The unknown concentration in samples can be determined by extrapolation from this standard curve.

Assay Procedure

The 20x assay buffer concentrate provided with the kit was diluted with 950 ml of distilled water. This 1x assay buffer solution was used to dilute or reconstitute all other reagents in the kit and samples. The buffer was placed in a warm water bath for approximately 30 minutes so that crystals were dissolved. The standard peptide was centrifuged and diluted

with 1 ml of 1x assay buffer and then vortexed. This was the stock solution of standard peptide with a concentration of 1000 ng/ ml. The solution was allowed to sit at least 10 minutes at room temperature (20-23 °C) to completely dissolve. Stock solution was centrifuged and vortexed immediately before use. Kisspeptin standard solutions were prepared in a serial dilution of the stock giving rise to 100, 10, 1, 0.1 and 0.01 ng/ml standards.

Primary antibody was rehydrated with 5 ml of 1x assay buffer and was mixed thoroughly. Biotinylated peptide was rehydrated with 5 ml of 1x assay buffer and was mixed thoroughly. Positive control was rehydrated with 200 µl of 1x assay and was mixed thoroughly. The assay procedure was then started as follows:

Wells A-1 and A-2 of the immunoplate were left empty as blank. 50 µl of 1x assay buffer was added into wells B-1 and B-2 as total binding. 50 µl of prepared peptide standards were added into wells from C-1 and C-2 to G-1 and G-2. Standards were added in duplicate. 50 µl of rehydrated positive control were added into wells H-1 and H-2.

50 µl of prepared samples were then added into their designated wells. 25 µl of rehydrated primary antibody was added into each well except the Blank well and it was followed by addition of 25 µl of rehydrated biotinylated peptide into each well except the blank well. The immunoplate was then sealed with the acetate plate sealer (APS) and incubated with orbital shaking at 300-400 rpm for 2 h at room temperature (20-23 °C). SA-HRP vial was centrifuged at (3,000-5,000 rpm, 5 seconds) and 12 µl of SA-HRP was pipetted into 12 ml of 1x assay buffer to make SA-HRP solution and vortexed thoroughly. APS was then removed from the immunoplate, contents of the wells were discarded and each well was washed with 350 µl of 1x assay buffer. The buffer was then discarded and the plate was inverted and then blot dried. This washing was repeated four times. Next, 100 µl of SA-HRP solution was added into each well, the immunoplate was resealed with APS and

incubated for 1 hour at room temperature (20-23 °C) with orbital shaking at 300-400 rpm. After incubation APS was removed from the immunoplate, plate was washed and blot dried 4 times with 1x assay buffer. 100 µl of tetramethylbenzidine (TMB) substrate solution was then added into each well with orbital shaking at 300-400 rpm. The immunoplate was then covered with APS and aluminium foil to protect from light and incubated for 1 h at room temperature (20-23 °C). Next, 100µl 2N HCl was added into each well to stop the reaction. The colour in the well changed from blue to yellow. Absorbance was read on a microplate reader (BIO RAD, Tokyo, Japan) at 450 nm within 20 minutes.

Calculations of Results

Standard curve was constructed by plotting the known concentrations of standard peptide on the log scale (X-axis), and its corresponding O.D. reading on the linear scale (Y-axis). The concentration of standard peptide is quantified using a four parameter equation. The standard curve showed an inverse relationship between peptide concentrations and the corresponding absorbance. The standard curve was of a reverse sigmoidal shape.

Statistical Analysis

Statistical comparisons for the mean plasma levels of kisspeptin, in vitro levels of testosterone and inhibin were made by one way and two way ANOVA, and paired t test. All data are presented as mean (± SEM). Statistical analyses were carried out using Graphpad Prism software version 6 (www.graphpadprism.org) (GraphPad Software. Inc, La Jolla, CA, USA). Results were considered statistically significant at $P < 0.05$.

Chapter 1

Kiss1 and Kiss1 Receptor Expression in the Rhesus Monkey Testis: a Possible Local Regulator of Testicular Function

ABSTRACT

Kisspeptin, a *Kiss1* gene product, stimulates the GnRH neurons in the hypothalamus, but some recent studies have also suggested a direct effect of kisspeptin on peripheral organs including the gonads. In this study we aimed to localise kiss1 and its receptor (kiss1r) in the non-human primate (rhesus monkey) testis.

Expression of kiss1 and kiss1r was detected in the testicular tissue of adult rhesus monkey using immunohistochemistry and reverse transcriptase PCR analysis (RT-PCR). Double label immunohistochemistry was used to localize kiss1 with germ cell marker T4 using specific antibodies for both. Dual immunohistochemistry was also used to colocalize kiss1r and inhibin (a Sertoli cell marker). Immunohistochemical analysis of testicular tissue from 6 adult rhesus monkeys was conducted on formaldehyde fixed tissues using 5μ thick sections. Reverse transcriptase PCR was carried out by using the gene specific primers to find the expression of kiss1 and kiss1r mRNAs in the testicular tissue of adult rhesus monkey. Kiss1 immunoreactivity was localised to spermatocytes and spermatids and Kiss1r was observed in spermatocytes and Sertoli cells. By using the double-label immunohistochemistry, kiss1 and T4 (sperm marker) were co-localized in spermatids and kiss1r and inhibin (Sertoli cell marker) were co-localized in Sertoli cells. The gene specific primers for *Kiss1* and *Kiss1r* mRNAs in reverse transcriptase polymerase chain reaction (RT-PCR) showed expression of *Kiss1* and *Kiss1r* in the adult rhesus monkey testis.

The present results indicate the presence of kiss1 and kiss1r first time in adult primate testis. These results suggest a possible autocrine/paracrine role of kisspeptin in non-human primate testis. The expression profile of kiss1 and its receptor in the testis suggests a possible direct involvement in a regulatory network involved to regulate spermatogenesis.

INTRODUCTION

Hypothalamic-pituitary-gonadotrophic axis (HPG) is regulated by many central and peripheral signals which target the GnRH neurons (Terasawa and Fernandez, 2001; Tena-Sempere and Huhtaniemi, 2003). Kisspeptin-Kiss1r system has received particular attention as a possible mechanism of GnRH activation (Plant, 2006; Popa et al., 2008). Kisspeptins are encoded by the Kiss1 (non-human)/ KISS1 (human) gene. Kiss1 mRNA is translated into a 145-amino acid peptide, from which a 54-amino acid protein and other shorter fragments are cleaved; all of these are collectively called as kisspeptins. All kisspeptins express similar affinity for the kisspeptin receptor (Kiss1r) (West et al., 1998; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Gottsch et al., 2009). Kisspeptin belongs to the RF amide family of peptides that possess an Arg- Phe- amide sequence motif at their C-terminus (Clements et al., 2001). Kiss1 acts through a G protein couple receptor, GPR54 (also named as AXOR12 or hOT7T175), now termed as KISS1R/ Kiss1r/ (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Gottsch et al., 2009).

Kisspeptin has diverse roles in a number of physiological processes including metastasis suppression, regulation of reproduction and metabolism (Mead et al., 2007; Clarke and Caraty 2013; Colledge et al., 2013). Kiss1 peptides play a significant role in regulating the reproductive function (de Roux et al., 2003; Seminara et al., 2003; Mead et al., 2007). Kiss1 stimulates the gonadotrophin secretion in many species and thus is an important regulator of HPG axis (Gottsch et al., 2004; Matsui et al., 2004; Navarro et al., 2004; Dhillo et al., 2005; Shahab et al., 2005; Caraty et al., 2007; Oakley et al., 2009). Kisspeptin elicited GnRH secretion by rat hypothalamic explants (Thompson et al., 2004; Castellano et al., 2005). Intracerebroventricular administration of kisspeptin increased GnRH in

cerebrospinal fluid in the sheep (Messager et al., 2005) and in hypophysial portal blood (Smith et al., 2011).

In addition to central axis, kisspeptin has now been discovered to play roles in peripheral organs as well. Kiss1 and Kiss1r expression and function has been found in many peripheral organs including neocortex of fetal adrenals (Nakamura et al., 2007), pancreatic islet endocrine cells (Hauge-Evans et al., 2006), aorta, coronary artery, umbilical vein (Mead et al., 2007), rat and human adipose tissue (Brown et al., 2008; 2009: Cockwell et al., 2012).

Altough, central Kiss1 signalling has an established role in regulating the reproductive axis, interestingly, new evidences suggest that Kiss1 may also act upon reproduction at the level of gonads. Expression of Kiss1 and Kiss1r was reported in the adult rat ovary throughout the whole estrous cycle (Castellano et al., 2006). Kisspeptin plays a role also in modulating the uterine implantation (Hiden et al., 2007).

High expression of KISS1 and a low to moderate KISS1R expression was observed in human testis (Kotani et al., 2001: Ohtaki et al., 2001). However, it is not known which specific cells (Sertoli cells, Leydig cells, germ cells and peritubular cells) in the testis express Kiss1 and Kiss1r.

The testes are primary reproductive organs in the male, surrounded by tunica albuginea (Kerr et al., 2006). The mammalian testis is considered to be divided into two compartments, tubular compartment consisting of seminiferous tubules and an interstitial compartment present between the tubules (Kerr et al., 2006; Weinbauer et al., 2010). Tubular compartment contains the germ cells and somatic cells; the peritubular cells and the Sertoli cells (Weinbauer et al., 2010). Interstitial compartment contains blood vessels,

Leydig cells and macrophages (Sharpe, 1984). Sertoli cells built tight junctions between each other called the blood-testis-barrier. This blood-testis-barrier divides the seminiferous epithelium into basal and adluminal reigons. Earlier stages of germ cells are located within the basal region and the maturing germ cells are located in the adluminal reigon (Kerr et al., 2006).

Testis performs two main functions; spermatogenesis, production of mature sperm from spermatogonia (Kerr and de Kretser, 1981; Waites and Gladwell, 1982; Sharpe, 1983) and steroidogenesis production of testosterone. Spermatogenesis takes place in the tubular compartment and testosterone production in the Leydig cells (Kerr et al., 2006). The functions of testis are regulated by a classical pituitary input or by a local regulation. Hypothalamus and pituitary have been demonstrated to be involved in regulating the both functions of testis. In turn, testicular hormones have also been known to carry out negative feedback regulation of the HPG axis (reviewed in Leutjens et al., 2005).

In addition to central endocrine regulation, it has been found that complex machinery of cells within the testis is also involved in controlling the testicular functions. The local factors may be paracrine and autocrine (Weinbauer and Wessels, 1999). These local factors are considered as regulators of pituitary hormone action and intrarcellular communication (Weinbaur et al., 2001). Local interactions also involve the interplay between the different testicular compartments (Weinbauer et al., 2010). Sertoli cells interact with germ cells through the secretion of many factors and participate in the local regulation of spermatogenesis. These factors include growth factors, cytokines, transferrin, connexion-43, plasminogen activator and many other molecules (Hneji et al., 1991; Skinner et al., 1991; Vliegen et al., 1993; Meachem et al. 2001; Benahmed, 1997; Brehm et al., 2007; Sridharan et al., 2007; Boepple et al. 2008). Germ cells have also been evidenced to control

the activity of Sertoli cells by secreting factors (Skinner et al., 1991; Lonnerberg et al., 1992; Parvinen et al., 1992; Chen et al., 1997; Franca et al., 1998). The germ-germ cells interaction is also an important part of testicular paracrinology. Germ cells express many factors like OCT-4 transcription factor (Pesce et al., 1998), neurogenin 3 (Ngn3) (Yoshida et al., 2004), GFRa (Elling et al. 2006) and SALL4 (Phillips et al., 2010).

The testes express many neuropeptides including GHRH, CRH, ACTH and GnRH (Weinbaur et al., 2010). Gonadotropin inhibitory hormone (GnIH) peptide (RERP in mammals) expression was described in germ cells of rhesus monkey and hamster (McGuire and Bentley, 2010; Zhao et al., 2010). Kiss1 expression in human testis may provide a new paracrine player (Kotani et al., 2001). Peripheral kisspeptin administration in acyline clamped pituitary of rhesus monkey stimulated hCG dependent testosterone release (Irfan et al., 2008). Moreover, in adult male monkeys, continuous intravenous infusion of kisspeptin invariably exaggerated the plasma T concentrations for a given LH stimulus (Ramaswamy et al., 2007). These factors point towards an involvement of Kiss1-Kiss1r signaling in regulating the testicular functions. Recently the presence of KISS1 and its receptor have been demonstrated in mature human spermatozoa (Pinto et al., 2011). However, there is still a lack of information about precise location of action of Kiss1 in the testis. The previous studies utilized only RNA expression techniques like reverse transcriptase PCR (RT-PCR) and in situ hybridization which do not highlight the morphology of the testis. Characterization of specific cells within the testis and specifically of germ cell types may have an important role in unveiling the kisspeptin as a direct local regulator of testis. The present study was therefore, designed to characterize the expression of Kiss1 and Kiss1r in the testis of non-human primate using rhesus monkey as a model using double label immunohistochemistry and reverse transcriptase PCR.

MATERIALS AND METHODS

Animals

A total number of eleven adult intact male rhesus monkeys (Macaca mulatta), 7-9 years old, weighing 8.3 -14.7 kg were used in this study. Their testicular volumes were 19.1-61.4 ml. The animals were housed in individual cages and maintained under semi-controlled environmental conditions of temperature (25±3°C) and light (06:00-18:00) in the Primate Facility of the Qauid-i-Azam University. Standard monkey diet supplemented with fresh fruits (09:00-09:30 h), boiled eggs (11:00 h) and bread (13:00-13:30 h) was provided daily. Water was available *ad libitum*. All the animal experiments were approved in advance by the Departmental Committee for Care and Use of Laboratory Animals.

Fluorescence Immunohistochemistry

Tissue Collection

For immunohistochemical studies, testes were obtained from 6 adult intact male rhesus monkeys weighing 6.8-14.7 kg and their mean testicular volume was 45 ml ± 1. The monkeys were sedated with ketamine hydrochloride (Ketler, Astarapin, Germany) given at a dose of 10 mg/kg body weight (BW) intramuscularly and then euthanized with ketamine hydrochloride 25-30 mg/kg BW intravenous injection. These animals were euthanized for taking hypothalami to be used in another study (Wahab et al., 2010). After removing the tunica albugenia, about 100 mg testicular tissues were obtained from anterior pole of left testis of each animal. The testicular tissues were immediately transferred to sera fixative. The experiments were carried out during September 2009.

Tissue Fixation and Processing

For immunohistochemistry, testicular tissues were immersion-fixed in formaldehyde-alcohol-acetic acid fixative (60 ml absolute alcohol (Sigma-Aldrich GmbH, Steinheim, Germany), 30 ml of formaldehyde (Merck, Darmstadt, Germany) and 10 ml of glacial acetic acid (Merck) for 4-5 h. The tissues were then passed through graded alcohols and placed in cedar wood oil (Serva Electophoresis, GmBH, Heidelberg, Germany) until they appeared clear. Tissues were then washed twice (10 min) with benzol, placed in benzol+paraplast (1:1) (Fluka AG, Buchs, Switzerland) for 20 min and then washed 3 times (3 x 12 h) in Paraplast (Fluka AG, Buchs, Switzerland) at 60 °C to keep it in liquid state. Finally, the tissues were embedded in paraffin and sectioned at 5 µm on a Shandon Finesse 325 microtome (Thermo Electron Cooperation, Waltham, MA, USA).

Kisspeptin Immunohistochemistry

Eight 5 µm thick sections were taken for analysis from paraffinized testis tissue blocks of each animal. Paraffin embedded sections were de-waxed in xylene (Merck, Darmstadt, Germany), rehydrated through descending grades of alcohol and rinsed in distilled water. The sections were then washed with 0.1M Tris-buffered saline (TBS) (pH 7.4), mounted on superfrost slides (Santa Cruz Biotechnology, Heidelberg Germany) and dried over-night. Antigen retrieval was then performed using 1 M citrate buffer (pH 6.0) and heating in a microwave oven (2x3min) at 1000 W. The slides were then washed in TBS (3x10 min) and incubated in blocking solution containing 10% normal goat serum and 0.3% Triton X-100 (Merck, Darmstadt, Germany) in 0.1 M Tris-buffered saline (TBS) for 2 h at room temperature. The sections were then incubated in primary antibody for kisspeptin (GQ2) for 72 h at 4 °C at a dilution of 1:120,000. The sections were washed in TBS, incubated with secondary antibody donkey anti-sheep Alexa 488 for 2 h at room temperature, rinsed

in TBS, and then counterstained with 0.3% Sudan Black B (Santa Cruz Biothcnology Heidelberg Germany) to minimize autofluorescence. Sections were rinsed in TBS and then 0.1 M phosphate buffer. The sections were then mounted with a preparation of an anti-fade fluorescent medium (DAKO, Carpentaria, CA, USA) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; nucleic acid stain, Molecular Probes, Eugene, Oregon, USA). Sections were then coversliped and kept at 4 °C until examined.

Colocalization of Kisspeptin and T4

Eight 5 μm thick sections were taken for analysis from paraffinized testis tissue blocks of each animal. Paraffin embedded sections were de-waxed in xylene, rehydrated through descending grades of alcohol and rinsed in distilled water. The sections were then washed in 0.1M Tris-buffered saline (TBS) (pH 7.4), mounted on superfrost slides and dried over-night. Antigen retrieval was then performed using 1 M citrate buffer (pH 6.0) and heating in a microwave oven (2x3 min) at 1000 W. The slides were then washed in TBS (3x10 min) and incubated in blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in 0.1 M Tris-buffered saline (TBS) for 2 h at room temperature. These sections were then incubated in a cocktail of primary antibodies (GQ2 for kisspeptin and rabbit anti T4 for T4) for 72 h at 4 °C, both antibodies were at a dilution of 1:2000. The sections were then washed in TBS and incubated in cocktail of secondary antibodies (donkey anti-sheep Alexa 488 and goat anti-rabbit Alexa 546, both diluted 1:500) for 2 h at room temperature. Sections were rinsed in TBS, and then counterstained with 0.3% Sudan Black B to minimize autofluorescence. Following rinses in TBS and then in 0.1 M phosphate buffer, sections were mounted with a preparation of an anti-fade fluorescent medium and DAPI. Sections were then coversliped and kept at 4 °C until examined.

Kiss1r Immunohistochemistry

Eight sections were taken for analysis from paraffinized testis tissue blocks of each animal. 5 µm thick sections were de-waxed in xylene, rehydrated through descending grades of alcohol and rinsed in distilled water. The sections were then washed in 0.1M Tris-buffered saline (TBS) (pH 7.4), mounted on superfrost slides and dried over-night. Antigen retrieval was then performed using 1 M citrate buffer (pH 6.0) and heating in a microwave oven (2x3 min) at 1000 W. The slides were then washed in TBS (3x10 min) and incubated in blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in 0.1 M Tris-buffered saline (TBS) for 2 h at room temperature. The sections were then incubated in primary antibody for Kiss1r (Rabbit anti-AXOR12) for 72 h at 4°C at a dilution of 1:1000. The sections were washed in TBS, incubated with secondary antibody (goat anti-rabbit Alexa fluor 488) for 2 h at room temperature in a humid chamber, at a dilution of 1:500. They were then rinsed in TBS, and counterstained with 0.3% Sudan Black B to minimize autofluorescence. Following rinses in TBS and 0.1 M phosphate buffer, sections were mounted with a preparation of an anti-fade fluorescent medium and DAPI. Sections were then coversliped and kept at 4 °C until examined.

Colocalization of Kiss1r and Inhibin

Eight sections were taken for analysis from paraffinized testis tissue blocks of each animal. Paraffin embedded 5 µm thick sections were de-waxed in xylene, rehydrated through descending grades of alcohol and rinsed in distilled water. The sections were then washed in 0.1 M Tris-buffered saline (TBS) (pH 7.4), mounted on superfrost slides and dried over-night. Antigen retrieval was then performed using 1 M citrate buffer (pH 6.0) and heating in a microwave oven (2x3 min) at 1000 W. The slides were then washed in TBS (3x10 min) and incubated in blocking solution containing 10% normal goat serum and 0.3%

Triton X-100 in 0.1 M Tris-buffered saline (TBS) for 2 h at room temperature. The sections were then incubated in a cocktail of primary antibodies (GPR54 raised in rabbit 1:1000 and inhibin raised in mouse 1:100) for 72 h at 4 °C. The sections were then washed in TBS and incubated in cocktail of secondary antibodies (goat anti-rabbit Alexa 488 and goat anti mouse Alexa 546, both diluted 1:500) for 2 h at room temperature. These were rinsed in TBS, and counterstained with 0.3% Sudan Black B to minimize autofluorescence. Following rinses in TBS and 0.1 M phosphate buffer, these sections were mounted with a preparation of an anti-fade fluorescent medium and DAPI, coversliped and kept at 4°C until examination.

Microscopy

Slides were viewed under 20x and 40x objective lenses on a Zeiss AX10 microscope with fluoresence lamps (Carl Zeiss, Inc, GMBH, Jena, Germany). Fluorescence images of single or double labeling were created using Zeiss image analysis software by using fluorescence filters: Alexa fluor 488, Alexa fluor 546 and texas red 546. Single label fluorescence images for kisspeptin and inhibin, and kisspeptin and T4 were merged to get double labeled images.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Tissue Collection

For measurement of mRNA, by RT-PCR, approximately 100 mg of testicular tissues were collected from biopsied tissues of each monkey under sterile surgical conditions. These tissues were immediately frozen in liquid nitrogen and kept at -70 °C until used for RNA extraction.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from testicular tissue using the TRIzol reagent (Invitrogen Life Sciences, Carlsbad, CA, USA) and reverse transcribed with a First Strand cDNA Synthesis kit (Fermentas, Osaka, Japan). The kiss1 and kiss1r primers were designed to target a segment comprised within the cloned cDNA. Primer sequences were selected as described by Wahab et al (2010). The primer sequences were synthesized by Fermentas and ThermoFisher Scientific (Osaka, Japan) and were as follows:-

Kiss1r: forward - 5′-CTCGCTGGTCATCTACGTCA-3′,

reverse -5′ -CGAACTTGCACATGAAATCG-3′

Kiss1: forward -5′-CTGGAATCCCTGGACCTCTC-3′

reverse 5′-TTGTAGTTCGGCAGGTCCTT-3′.

Amplification of cDNA was carried out in a reaction mixture of 50 µl. The mixture consisted of 3 µl of cDNA, 0.7 µl Taq polymerase, 35 µl RNAse free water, 2.5 µl of each forward and reverse primer, 5 µl reaction buffer and 1.3 µl dNTP. PCR conditions were: 10 min at 95 °C, 40 cycles of 30 seconds (s) at 95 °C, 1 min at 60 °C, 30 s at 72 °C using a thermocycler (Whatman Biometra GmBH, Goettingen, Germany). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as internal control.

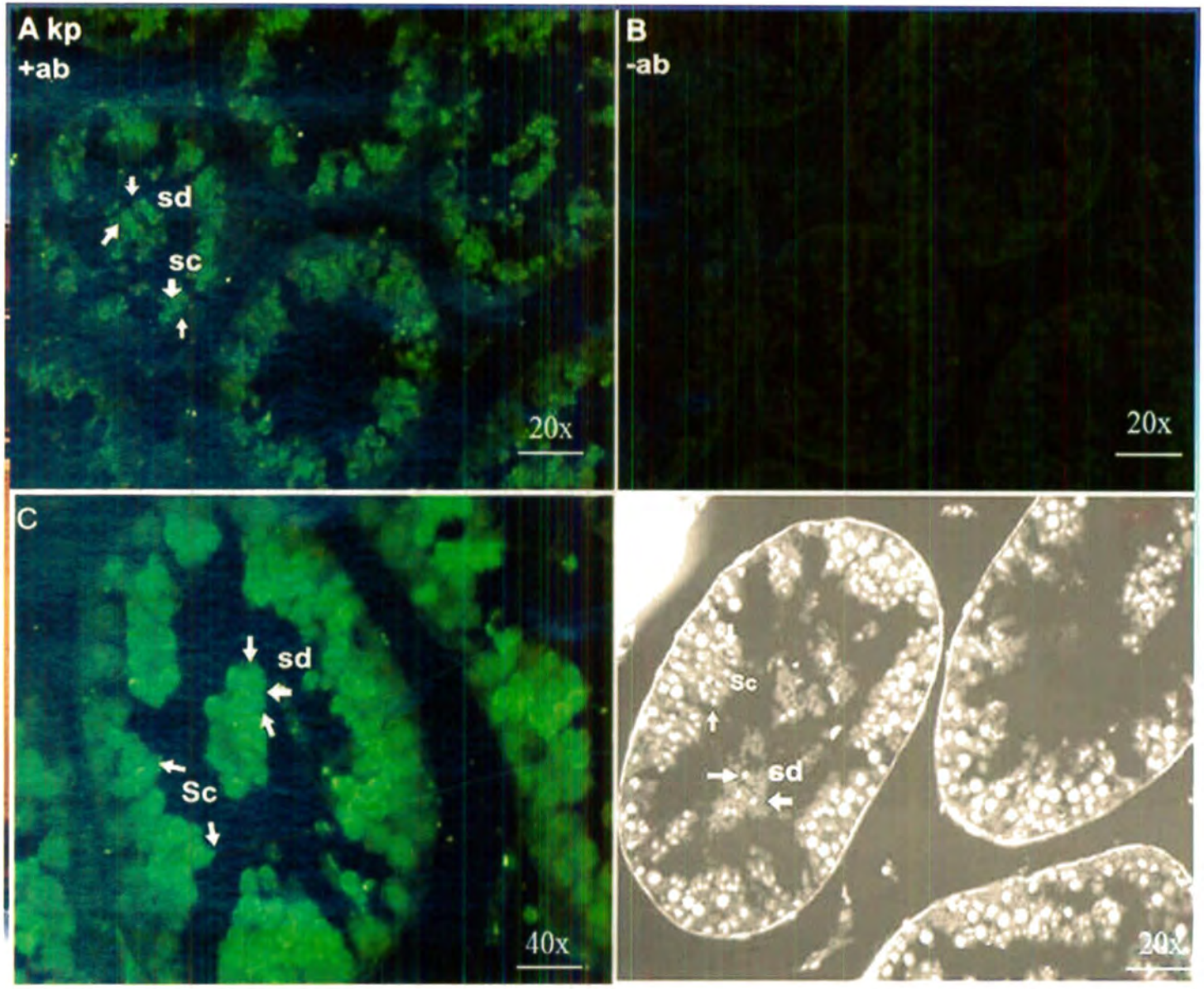

Figure 1.1 Expression of kisspeptin (kp) in seminiferous tubules of the adult rhesus monkey. Kisspeptin (kp) immunoreactivity was observed in spermatides (Sd) and spermatocytes (Sc) (A, 20x). Antibody omitted control showed no expression (B, 20x). A 40x maginification of a tubule is shown in (C). DAPI was used for cellular nuclei staining (D, 20x).

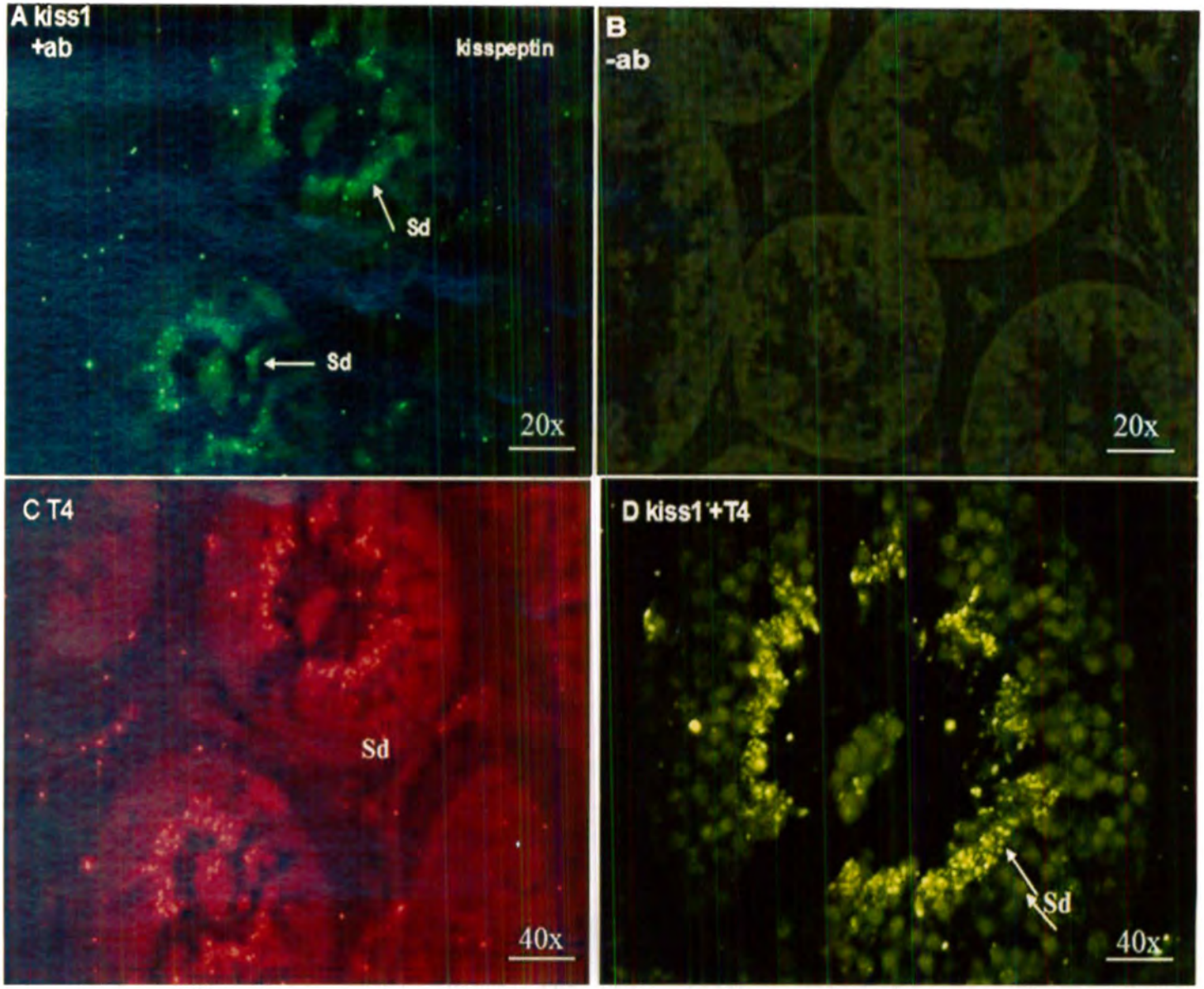

Figure 1.2. Expression of kisspeptin (kp) (A, 20x) and germ cell marker T4 (C, 20x) in the adult rhesus monkey testis. Both kisspeptin and T4 were localized in and spermatids (Sd). No immunoreactivity was found in antibody omitted controls (B). Dual-label cells stand out in yellow when images were merged (D, 20x).

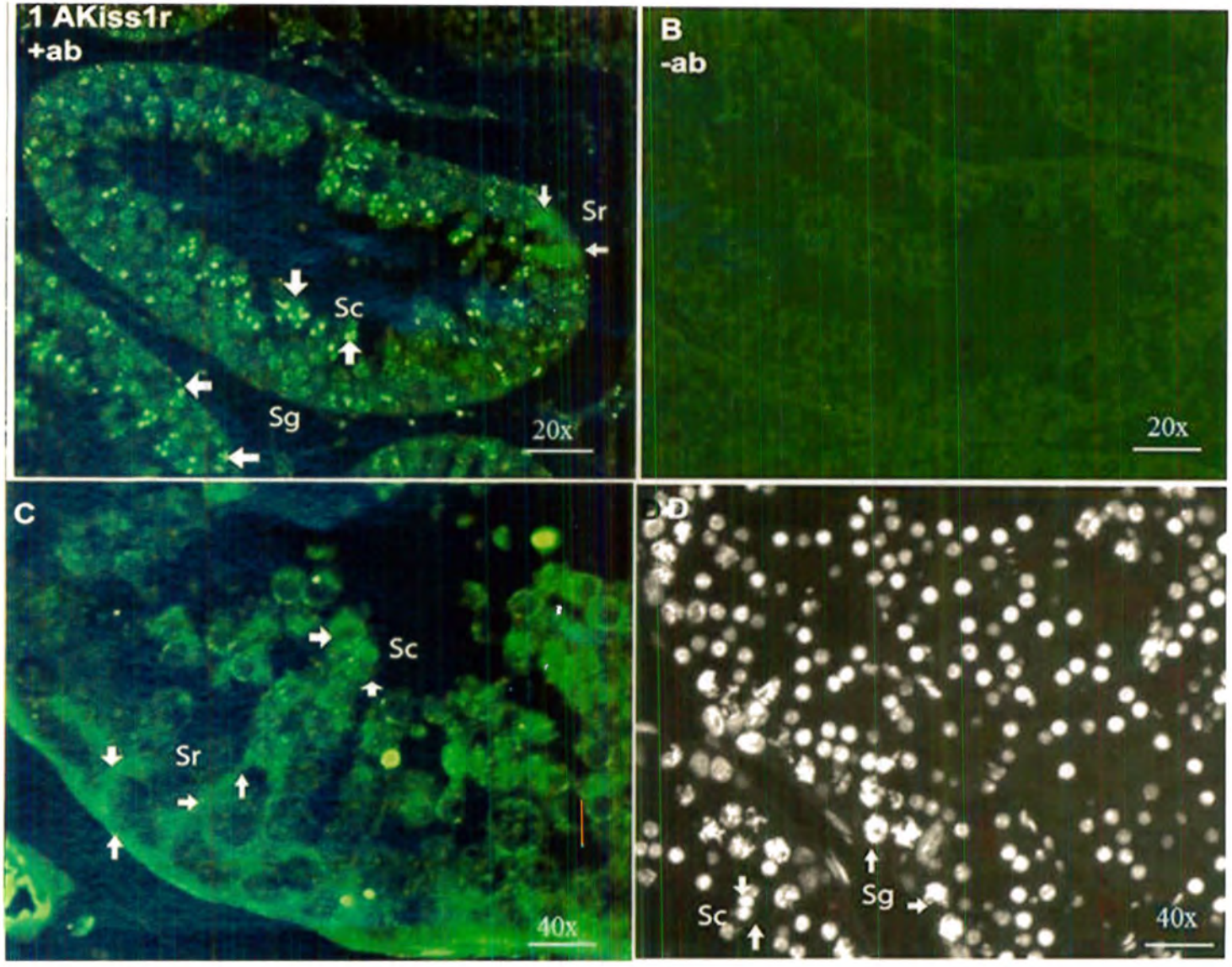

Figure 1.3. Expression of Kiss1r in seminiferous tubules of the adult rhesus monkey. Kiss1r immunoreactivity was observed in spermatogonia (Sg), spermatocytes (Sc) and Sertoli cells (Sr) (A, 20x). Antibody omitted control (B, 20x) showed no expression. Sertoli cell immunreactivity is shown in a separate section (C, 40x), DAPI was used for cellular nuclei staining (D, 40x).

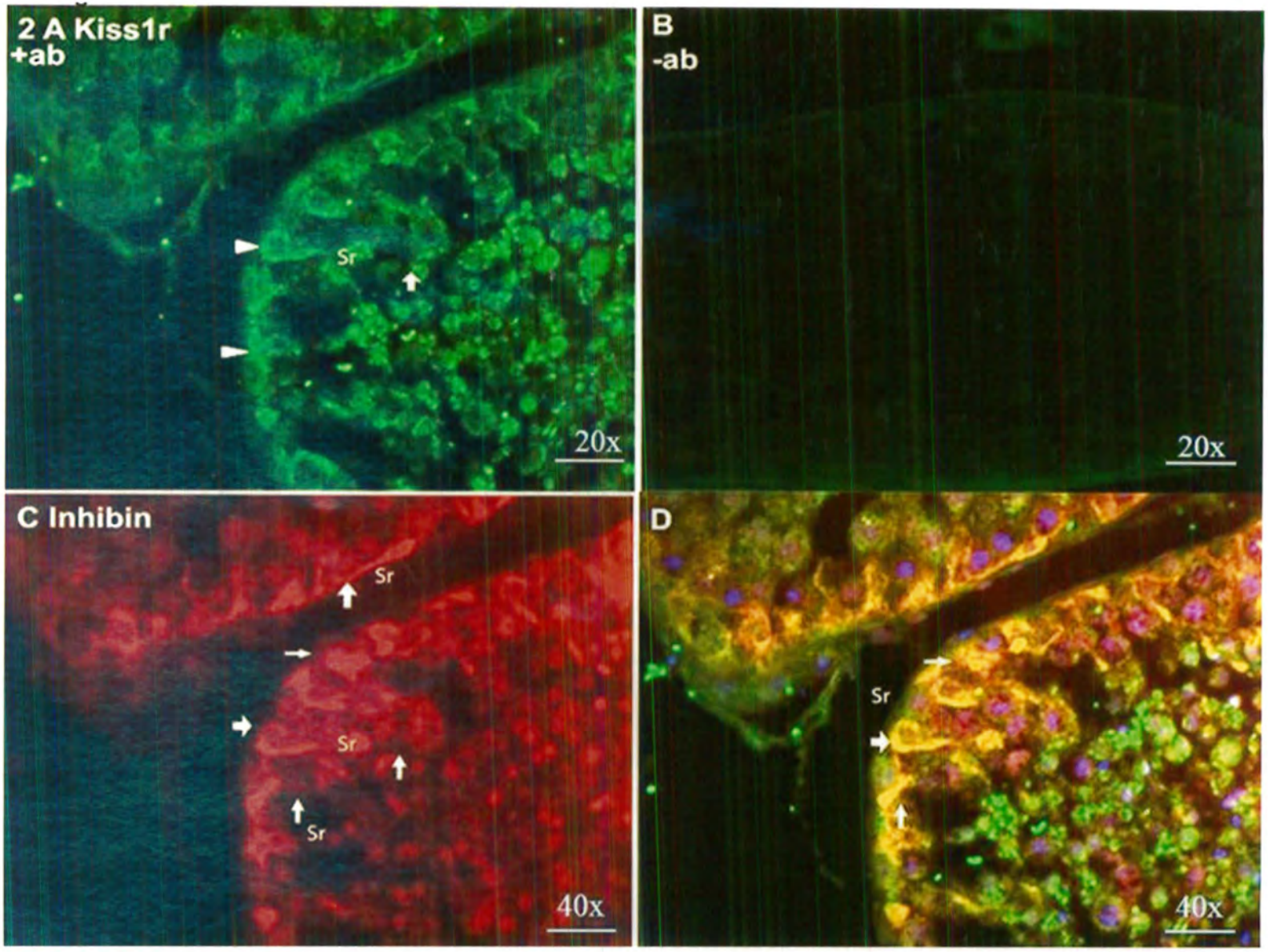

Figure 1.4. Expression of Kiss1r (A, 20x) and Sertoli cell marker, inhibin (C, 20x) in seminiferous tubules of the adult rhesus monkey. Both Kiss1r and inhibin were localized in Sertoli cells. No immunoreactivity was found in antibody omitted controls (B, 20x). Dual-label cells stand out in yellow when images were merged (D, 20x).

Although, Kisspeptin and Kiss1r were not observed in Leydig cells but for assuring this finding, they were double labeld with Leydig cells specific marker antibodiy 17βHSD. Kisspeptin and Kiss1r expression was not found in the Leydig cells in double label.

Kiss1 and *Kiss1r* mRNA Expression in the Rhesus Monkey Testis

RT-PCR confirmed *Kiss1* and *Kiss1r* expression in the adult monkey testes (Figure 1.5), with no signal found for the cDNA negative controls. Clear bands of the expected size (250 bp for *Kiss1* and 171 bp for *Kiss1r)* were observed. Gleyceraldehyde 3 Phosphate (GAPDH) was used as an internal control.

Above data have been recently published (Appendix 1).

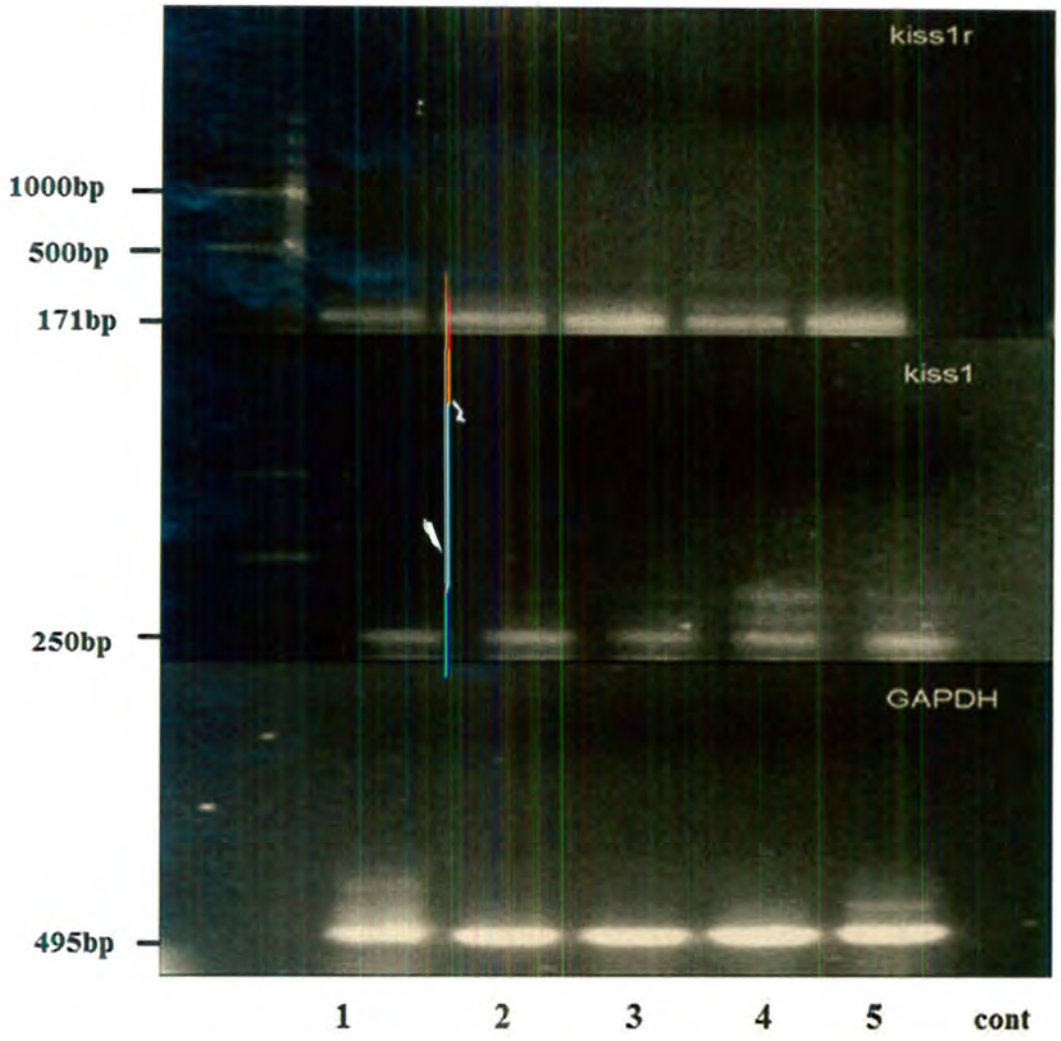

Figure 1.5. Expression of the mRNAs of kisspeptin and *Kiss1r* in the adult rhesus monkey testis. RT-PCR analysis from five testes samples are presented. Amplicons of 250 bp (kisspeptin) and 171 bp (*Kiss1r*) were obtained, cDNA negative controls of the respective genes showed no bands of corresponding size of positively expressing genes. Amplification of GAPDH served as an internal control.

DISCUSSION

The present work provides an evidence for the expression of Kiss1 and its receptor in adult rhesus monkey testes and localizes their production to specific cell types within the seminiferous tubules. The immunohistochemical analysis showed the localization of both Kiss1 and Kiss1r in germ cells and Kiss1r in Sertoli cells. Kiss1 and Kiss1r expression were notably absent in Leydig cells. Our immunohistochemical data were confirmed in our RT-PCR studies where a clear detectable mRNA expression of both kiss1 and kiss1r was observed in the testicular tissue. Present findings are in line with observations of an earlier study where kiss1 and Kiss1r expression was demonstrated in the human testis (Kotani et al., 2001). These data suggest that Kiss1 may act as a paracrine/autocrine factor, during primate spermatogenesis. Accordingly, Kiss1 that is produced by germ cells (spermatocytes) could act in an autocrine manner and/or also modulate Sertoli cells activity; communication between germ-germ cells is also possible. Thus, Kiss1 produced in spermatocytes and spermatids could act on Sertoli cells.

Process of spermatogenesis in the seminiferous tubules has been found to be under the regulation of both, Sertoli cells and germ cells, via physical interaction and secretion of biological factors. It is well established that Sertoli cells act to modulate spermatogenesis through various factors like IGF-I, IGF-2, TGF-α, TGF β, basic FGF, interleukins (Skinner et al., 1991). In turn, the capacity of the germ cells to modulate Sertoli cell activity has also been postulated (Huleihel and Lunenfeld, 2004). Androgen binding protein, plasminogen activator, and FSH-dependent cAMP production by Sertoli cells have been shown to be affected by germ cells (Skinner et al., 1992; Skinner et al., 2009; Huleihel and Lunenfeld, 2002). In this regard, germ cells have been shown to secrete follistatin (Ogawa et al., 1997; Meinhardt et al., 1998) and 4.1G a novel membrane skeletal protein (Terada et al., 2010).

Our findings suggest that the germ cells can also communicate with Sertoli cells with kisspeptin signalling. While the physiological significance of Kiss1 signalling within germ cells and between the germ cells and Sertoli cells remains to be determined. The recent observation that kisspeptin and Kiss1r have been localized to mature human spermatozoa would strongly suggest so (Pinto et al., 2011). It has been shown that changes in sperm motility are induced by Kiss1 which is reduced in the presence of the Kiss1r antagonist. These observations suggest that Kiss1 acting via Kiss1r, may activate different signal transduction pathways leading to its modulatory role on sperm movement.

In summary, present results based on immunohistochemical and RT-PCR data clearly demonstrate the presence of both Kiss1 and its receptor in the seminiferous tubules of the adult monkey testis. Expression of Kiss1 and Kiss1r mRNA was also evident in the testis. The presence of Kiss1 and its receptor in the testis suggests a possible direct involvement kisspeptin signaling in a regulatory network for testicular function The fact that Kiss1 and Kiss1r are restricted to seminiferous tubules suggests a paracrine/autocrine role of Kiss1 signaling related to spermatogenesis.

Chapter 2

Effect of Kisspeptin Challenge on Testosterone and Inhibin Secretion from *in Vitro* Testicular Tissue of Adult Male Rhesus Monkey (*Macaca mulatta*)

ABSTRACT

Kisspeptin regulates the reproductive function at the level of GnRH but, as kisspeptin expression has also been found in gonads, so a direct role of kisspeptin in reproductive function is being explored. The objective of this study was to find a dose and time related effect of kisspeptin on testosterone and inhibin secretion in an in vitro culture of testicular tissue (100 mg fragments) of adult male rhesus monkey (n=5). Kisspeptin (1, 10, 100, 1000 pM) was incubated to a culture of testicular tissues of adult male rhesus monkey and medium for hormone measurement was collected after 30, 60 and 120 minutes. 10 IU hCG and 50 ng FSH were incubated to the culture for 180 min and 120 min respectively for checking testicular cells ability to secrete testosterone and inhibin, respectively, in vitro. Kisspeptin did not significantly ($P<0.05$) increase the testosterone levels at any dose. However, one way ANOVA at pooled doses indicated that the testosterone levels increased after 120 minutes as compared to 30 and 60 minutes of incubation. This suggests an independent effect of time. hCG significantly ($P<0.05$) increased testosterone concentration compared to the basal groups. Effect of in vitro kisspeptin incubation in testicular tissue fragments in the culture medium was also investigated for a change in inhibin concentration. Kisspeptin had no effect on inhibin concentration at any dose studied. Paired t test showed an independent effect of time as inhibin concentration decreased after 120 minutes of incubation. FSH (50 ng) treatment to testicular tissue fragments in culture caused a significant ($P<0.05$) rise in inhibin. Kisspeptin do not affect the testosterone and inhibin secretion from the testis of adult monkey. We therefore concluded that kisspeptin has no role in testicular regulation related to testosterone and inhibin release but kisspeptin may have other roles in testicular regulation.

INTRODUCTION

Kisspeptins are derived from Kiss1 gene and are structurally related peptides which contain a common Arg-Phe-NH2 motif at their C-terminal. Kisspeptin act through G protein-coupled receptor (Kiss1r) (Lee et al., 1999; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). This signalling system has been identified as a vital regulatory part of the HPG axis. Kisspeptin affects the gonadotrophic axis via Kiss1r as in Kiss1r null mice, kisspeptin fails to stimulate LH release (Messager et al., 2005). Kisspeptin administration to rodents or primates stimulates LH and FSH release and this effect is mediated at the level of GnRH because GnRH antagonists block the stimulation of the gonadotropin by kisspeptin (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Navarro et al., 2004; 2005; Thompson et al., 2004; Kinoshita et al., 2005; Dhillo et al., 2005; Messager et al., 2005; Shahab et al., 2005; Plant et al., 2006). Continuous peripheral administration of kisspeptins to monkeys and rodents desensitized the Kiss1r (Seminara et al., 2006; Thompson et al., 2006; Ramaswamy et al., 2007). Chronic infusion of kisspeptin to adult rats leads to significant degeneration of the testes, germ and Sertoli cells (Thompson et al., 2006).

Kiss1/Kiss1r system is not only involved in signalling cascades at central level but has also been evidenced to play role in peripheral organs as well. Kiss1r protein is expressed in the neocortex of fetal adrenals and increases aldosterone production in fetal neocortex adrenal cells (Nakamura et al., 2007). Pancreatic islet endocrine cells express high levels of Kiss1 and Kiss1r and Kiss1 infusion to mouse and human islets caused stimulation of glucose-induced insulin secretion (Hauge-Evans et al., 2006). Kiss1 also acts as vasoconstrictor (Mead et al., 2007). Although, reproductive axis is mainly regulated by central Kiss1 signalling but evidences also suggest that Kiss1 can affect the reproduction while acting through pituitary or gonads. Expression of kisspeptin in different peripheral tissues is an

indication of a direct effect of kisspeptin in the respective organ. Gutierrez-Pascal et al. (2007) treated pituitary cells with increasing kisspeptin-10 concentrations and observed a kisspeptin induced dose-related LH secretion. Navarro et al. (2005) also found a dose dependent effect of kisspeptin on LH secretion in cultured pituitary cells. Cultured ovine pituitary cells exhibit a two-fold increase in LH secretion after incubation with kisspeptin (Smith et al., 2006). In this regard, expression of *Kiss1* and *Kiss1r* was reported in the adult rat ovary (Castellano et al., 2006). Kisspeptin from the oviduct has been suggested to be involved in the prevention of ectopic (tubal) implantation (Gaytan et al., 2007). *KISS1* and *KISS1R* expression has also been observed in human testis (Kotani et al., 2001: Ohtaki et al., 2001). It has also been observed that kisspeptin may enhance the hCG-stimulated testosterone in acyline-treated adult male rhesus monkeys (Irfan et al., 2008)

It is quite worth mentioning here that though kisspeptin has a profound effect on testosterone secretion through central axis but an effect of kisspeptin at testicular levels remains possible. However, no information is available on the effect on testis and its hormonal secretion. This study was therefore, designed to find if there was any role of kisspeptin on hormonal secretion from incubated testicular tissue of the adult male rhesus monkey.

MATERIALS AND METHODS

Animals

Five adult intact male rhesus monkeys (*Macaca mulatta*), 7-9 years old, weighing 8.3 -14.7 kg were used in this study. Their total testicular volumes ranged from19.1-61.4 ml. The animals were housed in individual cages and maintained under semi-controlled environmental conditions of temperature (25-28 °C) and light (06:00-18:00) in the Primate Facility of the Qauid-i-Azam University. Standard monkey diet supplemented with fresh fruits (09:00-09:30 h), boiled eggs (11:00 h) and bread (13:00-13:30 h) was provided daily. Water was available *ad libitum*. All animal experiments were approved in advance by the Departmental Committee for Care and Use of Laboratory Animals.

Testicular Tissue Collection

The testicular tissues were collected from the monkeys through biopsy which has been described in detail in General Material and Methods chapter. The tissues were de-capsulated, weighed and cut into five fragments of approximately 100 mg which were immediately transferred to fresh ice cold incubation medium containing 2 ml of Dulbecco's modified eagle's medium (DMEM PH 7.4) and 10% FBS. The tissues were then challenged with different kisspeptin doses.

Testicular Tissue Incubation

Analysis of the direct effect of kisspeptin on testosterone and inhibin secretion was carried out by using testicular slice incubation method which was done as previously described by Roulet et al. (2006) with slight modifications. Testicular slices were incubated for 1 h in 2 ml DMEM containing 1.2 g/l sodium bicarbonate, glucose (1 mg/ml), 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% FBS in 5 ml culture tubes under 5% CO_2 and 95% air at

32 °C in a CO_2 incubator (Innova CO-48, Edison, New Jersey). After incubation for 1 h, the media was replaced with medium containing kisspeptin at five different dose rates (no dose, 1 pM, 10 pM, 100 pM, 1000 pM). Aliquots of 100 µl medium were then taken from each tube after 30, 60 and 120 min of incubation and stored at -20 °C until assay. Then, all media was removed and replaced with media containing 10IU hCG (hCG; IVF-C, Jeonbuk-do, Korea). hCG incubation was used to evaluate the viability of the Leydig cells to secrete testosterone in the culture conditions. 100 µl aliquots of the medium were taken 3hr after hCG incubation. Again all the media was replaced with medium containing 50ng/ml FSH (Fetal Bovine Serum, Hyclone, Cramlington, UK) and 100 µl aliquots were taken after 2 h. FSH incubation was used to measure the viability of Sertoli cells to secrete inhibin.

Hormone Assays

Plasma testosterone and inhibin were determined by using specific enzyme linked immunoabsorbent assays. Testosterone and inhibin kits were purchased from the following companies, respectively, Amgenix Microlisa™ (San Jose, CA, USA), Beckman Coulter (Brea, CA, USA). The assays were performed according to the manufacturer's instructions. The sensitivity of the testosterone assay was 0.05 ng/ml and intra- and inter-assay coefficients of variation were both < 10%. Sensitivity of the inhibin assay was 2.6 pg/ml and the intra- and inter-assay coefficients of variation were < 6%.

Statistical Analysis

Statistical comparisons for the mean levels of testosterone and inhibin were made by one way and two ANOVA, and paired t test. One way ANOVA was followed with Turkey's comparison test. All data are presented as mean (± SEM). Statistical analyses were carried

out using Graphpad Prism software version 6 (www.graphpadprism.org) (GraphPad Software. Inc, La Jolla, CA, USA).

RESULTS

In Vitro Effect of Kisspeptin Treatment on Testosterone Secretion from Testicular Tissue of Adult Male Rhesus Monkey

The changes in individual and mean concentrations of testosterone (T) in the incubation medium from monkey testis fragments challenged with five different doses of kisspeptin (0 pM, 1 pM, 10 pM, 100 pM, 1000 pM) at three different time points are shown in Table 2.1 and Figure 2.1. Two- factor ANOVA, taking dose and time as main sources of variation demonstrated that there was no significant effect of kisspeptin dose, time and dose × time interaction on mean testosterone concentration. Kisspeptin did not significantly increase the testosterone levels at any dose, therefore, all the doses were pooled at every time point. Comparison of mean concentration of T after treatment of kisspeptin at three time points (all doses pooled) and post 3 h treatment of hCG is shown in Figure 2.2. One way ANOVA followed by post hoc test indicated that testosterone levels were increased at 120 min as compared to those at 30 and 60 minutes reflecting an independent effect of time, on in vitro testosterone release. The hCG challenge to the testicular tissue significantly increased T concentration when compared with the pooled levels observed at 30 and 60 minutes incubation.

In Vitro Effect of Kisspeptin Treatment on Inhibin Secretion from Testicular Tissue of Adult Male Rhesus Monkey

The changes in individual and mean concentrations of inhibin in the incubation medium released from monkey testis fragments challenged with five different doses of kisspeptin (0

pM, 1 pM, 10 pM, 100 pM, 1000 pM) at two different time points are shown in Table 2.2 and Figure 2.3. Two- factor ANOVA taking dose and time as main sources of variation demonstrated that there was no significant effect of kisspeptin dose and dose × time interaction on mean inhibin concentration. However, there was a significant effect of time suggesting probably an independent time related decrease of inhibin release from the testicular tissue. No effect of kisspeptin doses was observed, therefore all doses were pooled at every time point and the comparison is shown in Figure 2.4. Paired t test showed that mean inhibin levels observed after 120 minutes of incubation was significantly ($P<$ 0.05) decreased compared to 60 minutes values. FSH stimulated a significant ($^*P<$ 0.05) increase of inhibin release from incubated testis tissue samples as compared to the levels at 60 and 120 minutes.

Table 2.1. Changes in individual incubation medium concentrations of testosterone (ng/ml) observed at three time points under an *in vitro* treatment of adult rhesus monkey testis tissue with five different doses of kisspeptin and subsequently with hCG at one time point.

Dose (pM)	Animal	Post kisspeptin Time (min)			Post hCG Time (min)
		30	60	120	180
1000	M1	0.47	0.26	1.043	3.04
	M2	0.39	2.90	6.63	1.80
	M3	1.89	2.92	12.02	3.53
	M4	4.74	0.53	1.190	10.78
	M5	0.32	13.59	3.84	1.90
100	M1	0.74	1.053	0.34	3.76
	M2	0.41	5.11	4.42	1.84
	M3	3.08	1.76	6.65	4.04
	M4	9.64	0.17	1.79	10.85
	M5	7.08	3.11	3.71	12.17
10	M1	0.42	0.65	0.55	1.55
	M2	0.88	0.83	4.76	3.87
	M3	4.12	1.57	3.21	10.02
	M4	2.10	0.16	0.49	6.01
	M5	3.17	0.35	1.94	4.03
1	M1	0.73	1.54	0.89	3.33
	M2	0.70	1.12	6.20	2.03
	M3	5.32	1.24	10.08	7.33
	M4	2.02	0.74	1.85	27.38
	M5	0.16	1.53	2.75	2.87
0	M1	0.34	0.68	2.37	1.33
	M2	3.43	3.40	2.60	10.49
	M3	0.46	2.40	12.45	2.66
	M4	0.32	0.28	1.08	20.36
	M5	0.74	1.198	7.93	2.87

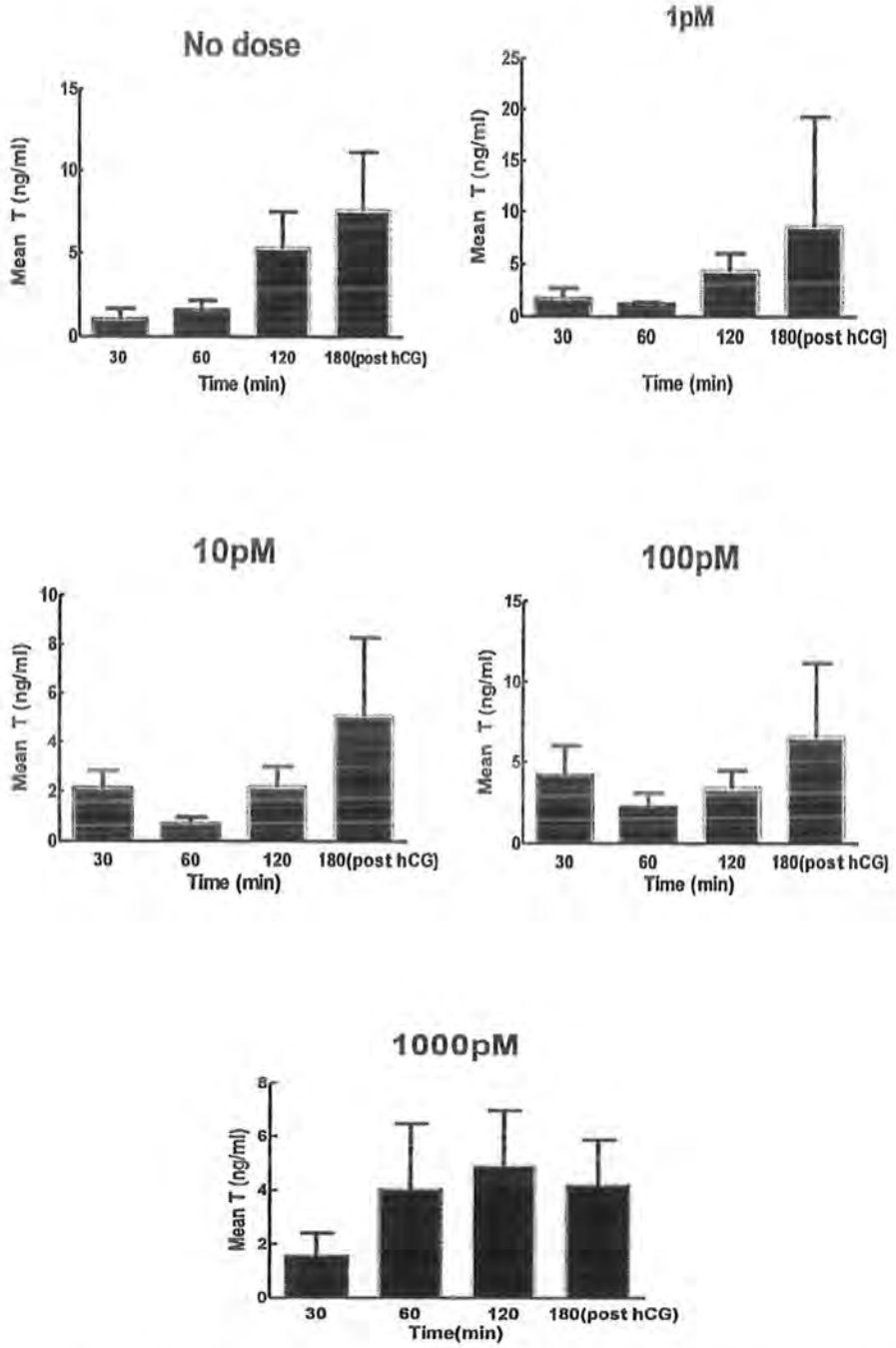

Figure 2.1. Effect of treatment of different kisspeptin doses on mean T concentration released from *in vitro* kept adult monkey testicular tissue in the incubation medium at three time points. Two- factor ANOVA taking dose and time as main sources of variation demonstrated that there was no significant effect of kisspeptin dose, time and dose × time interaction on testosterone concentrations.

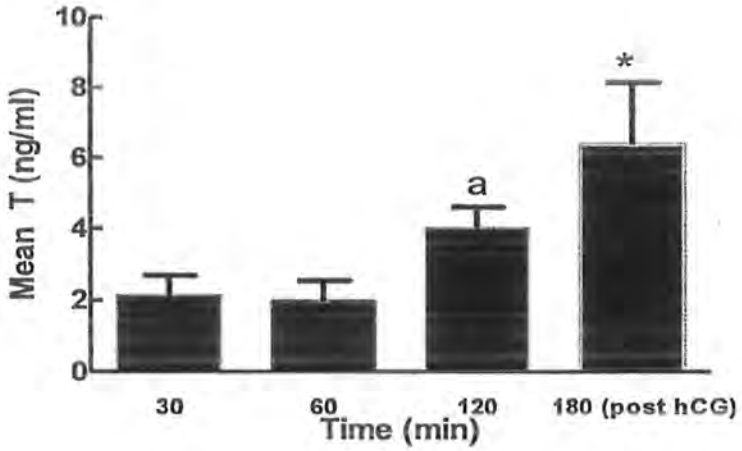

Figure 2.2. Comparison of change in mean T concentrations from adult monkey testicular tissue fragments incubated with kisspeptin with all doses pooled at every time point. On way ANOVA followed by post hoc test indicated that observed T levels were increased ($^{a}P<0.05$) at 120 minutes as compared to those at 30 minutes and 60 minutes reflecting an independent effect of time on *in vitro* testosterone release. hCG challenge increased T concentration significantly (*P <0.05) compared to the basal values (30 min and 60min).

Table 2.2. Changes in individual incubation medium concentrations of inhibin (pg/ml) observed at three time points under an *in vitro* treatment of adult rhesus monkey testis tissue with five different doses of kisspeptin and subsequently with FSH at one time point.

Dose (pM)	Animal	Post kisspeptin Time(min)		Post FSH Time(min)
		60	120	120
1000	M1	101	45.2	155
	M2	52	11.0	214
	M3	59.2	29.4	302
	M4	52.3	24.5	66.0
	M5	78.8	13.0	98.6
100	M1	90.3	41.2	212
	M2	88.3	24.1	340
	M3	165	97.4	84.5
	M4	327	48.4	81.5
	M5	38.2	11.5	77.1
10	M1	23.5	4.24	237
	M2	101	9.40	295
	M3	129	60.8	316
	M4	395	43.9	79.o
	M5	29.3	2.03	56.1
1	M1	34.0	127.	45.1
	M2	52.9	6.35	270
	M3	137	64.5	315
	M4	34.6	19.9	65.7
	M5	39.0	5.81	85.3
0	M1	263	43.6	44.1
	M2	91.3	174	220.0
	M3	178	78.4	334
	M4	73.5	43.0	29.9
	M5	13.7	2.21	88.7

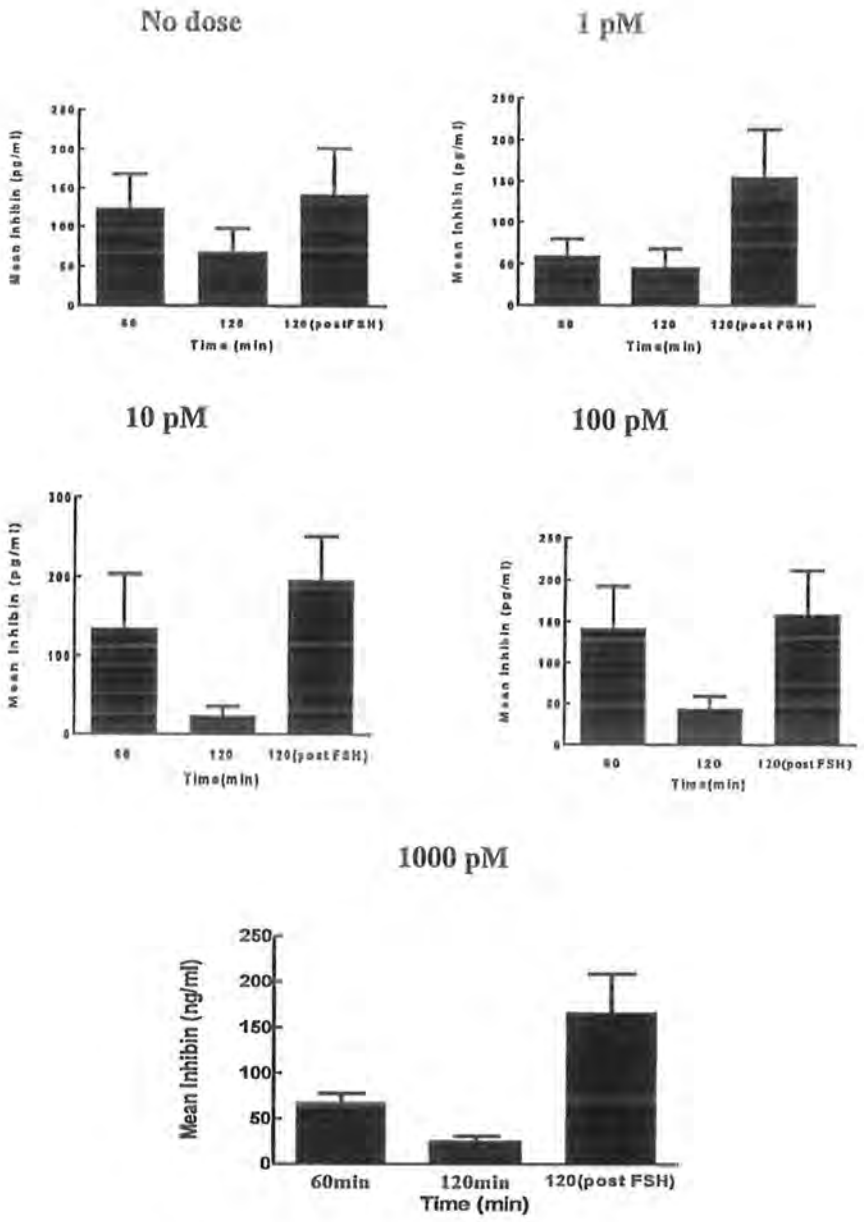

Figure 2.3. Effect of treatment of different kisspeptin doses on mean ± SEM inhibin concentration (n=5) released from *in vitro* kept adult monkey testicular tissue in the incubation medium at two time points. Two- factor ANOVA taking dose and time as main sources of variation demonstrated that there was no significant effect of kispeptin dose and dose × time interaction on inhibin concentration. However, there was a significant ($P < 0.05$) effect of time suggesting probably an independent time related decrease of inhibin release from the testicular tissue.

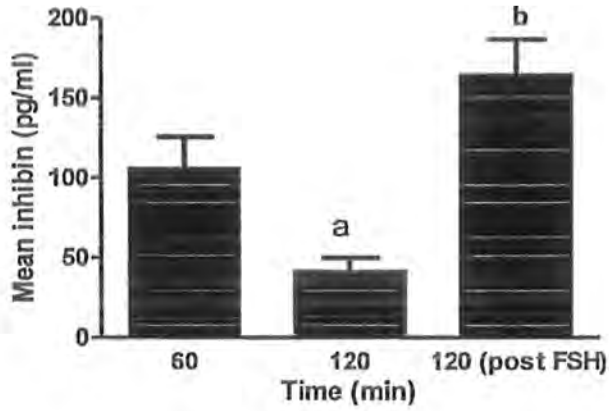

Figure 2.4. Changes in mean concentration of inhibin released from monkey testicular tissue after 60 and 120 minute of kisspeptin incubation and after 120 minutes of FSH incubation. Values are pooled at all doses of kisspeptin at two time points and at FSH treatment. Paired t test analysis showed that mean inhibin levels were significantly ($^{a}p< 0.05$) decreased at 120 minutes as compared to that at 60 minutes. FSH significantly (b) increased inhibin as compared to the levels observed at 60 ($^{b}p< 0.05$) and 120 minutes ($p< 0.0001$).

DISCUSSION

Kisspeptin and its receptor expression has been evidenced in testicular tissue (Kotani et al., 2001; Muir et al., 2001., Ohtaki et al., 2001), but surprisingly no further advances were made in finding a direct effect of kisspeptin on testicular tissue. Our own immunohistochemistry finding suggests the possibility of a direct/indirect effect of kisspeptin on Sertoli cells and Leydig cells in the monkey testis. The present study was therefore, designed to find such an effect of kisspeptin on testosterone and inhibin release from in vitro kept testicular tissue of the adult male rhesus monkey. Kisspeptin was incubated to a culture of testicular tissues of adult male rhesus monkey at different dose rates. The concentrations of testosterone and inhibin were determined in the culture media at different time points. Competence of Leydig cells and Sertoli cells in the testicular tissue was tested afterward with challenges of hCG and FSH, respectively. Two factor ANOVA revealed that kisspeptin administration did not affect the testosterone release in vitro from the monkey testicular tissue. The fact that lack of effect of kisspeptin on testosterone production in the paradigm of present study was not due to a problem in the capacity of the Leydig cells to release testosterone, was evidenced by ready release of testosterone by the hCG challenge. Since there was no dose effect, kisspeptin doses were pooled at every time point. One way ANOVA with post hoc test on the pooled data indicated that mean testosterone levels at 120 minutes of incubation were significantly increased as compared to those at 30 and 60 minutes. This likely reflected an independent effect of time on the release of testosterone from the testicular tissue. These results of lack of effect of kisspeptin on testosterone release are in accordance with our expression analysis where we did not find any expression of Kiss1r in the Leydig cells. As kisspeptin receptor was found in the Sertoli cells, therefore, we expected here a paracrine role of kisspeptin between Sertoli cell and Leydig cells through Sertoli cells secreted factors. Absence of kisspeptin

effect on testosterone secretion excludes the possibility of such role, however paracrine interactions related to other factors can't be ruled out. Other possibility could be that probably the dose administered was not sufficient to effect and activate testosterone synthesis. Use of comparatively increased doses may be able to produce a dose effect, but in vitro effects of kisspeptin have been found to produce contradictory results where kisspeptin was administered to pituitary in vitro cultures. Although in our study, kisspeptin did not affect the testosterone release but Irfan et al. (2008) found, hCG stimulated effect of kisspeptin on testosterone release in vivo. So it is possible that hCG stimulated kisspeptin may cause testosterone secretion also in in vitro setting.

Effect of in vitro kisspeptin administration to testicular tissue fragments was also investigated on inhibin release in the present study. Two factor ANOVA demonstrated that kisspeptin administration also did not affect the inhibin release in vitro from the monkey testicular tissue. FSH challenge stimulated inhibin release from the Sertoli cells in the testicular tissue which proved that lack of effect of kisspeptin on inhibin production was not due to a problem in capacity of the Sertoli cells to release inhibin. Since there was no dose effect, kisspeptin doses were pooled at every time point. Paired t test on the pooled data indicated that mean inhibin levels at 120 minutes of incubation were significantly decreased as compared to that at 60 minutes. This likely reflected an independent effect of time on the release of inhibin from the testicular tissue. Moreover, we did not use kisspeptin doses higher than 1000 pM and it is possible that higher kisspeptin doses are more effective in causing inhibin biosynthesis in Sertoli cells. Our data are somehow supported by a study by Thompson and colleagues (2004) who could not find a dose dependent effect of kisspeptin on LH secretion by rat pituitary fragment after 4 h incubation (Thompson et al., 2004).

In summary our results show that kisspeptin has no dose dependent effect on testosterone and inhibin secretion. Thus our data suggest that although Kiss1r is present in the testis of adult monkey but more probably it is involved in other kisspeptin actions which may not be directly related to testosterone and inhibin secretion. However, we cannot rule out that there are other testicular functions of kisspeptin which may not be relevant to testosterone and inhibin production but are directly or indirectly related to spermatogenesis.

Chapter 3

Study of Concentration Gradient of Kisspeptin in Testicular Fluids and Testicular Homogenate in Adult Male Rhesus Monkey *(Macaca mulatta)*

ABSTRACT

Kisspeptin is expressed at the gonadal level and has been implicated in mediating the gonadal functions, as has been demonstrated in ovarion tissue. Kisspeptin expression in testicular tissue has also been demonstrated but functional importance of kisspeptin in the testis remains to be elucidated. Considerable expression of kisspeptin in the testis raises the possibility of release of kisspeptin from the testicular cells. Therefore, in the present study, we hypothesized that if kisspeptin is expressed by specific cells of the adult monkey testis then there must be some levels of kisspeptin present in different testicular fluids or compartments. It was expected that levels of kisspeptin should be highest in the testis homogenate and there should be a decreasing concentration gradient of kisspeptin from testis homogenate to peripheral venous blood. To test the hypothesis, testicular vein blood, spermatic vein blood and peripheral venous blood were collected from five adult rhesus monkeys. Testis homogenate was also prepared from the biopsied testicular tissue. Kisspeptin concentration was then measured in the plasma from these blood samples and homogenate using specific enzyme linked immunoabsorbent assay. Additionally, kisspeptin release was also measured from the testis fragments in vitro, in order to compare the in vitro levels of kisspeptin with in vivo levels from testis. We found fairly detectable levels (0.29-18 ng/ml) of kisspeptin in testicular venous blood, spermatic vein, peripheral blood, testis homogenate and testicular incubation medium, but no significant variation was found among them. The kisspeptin levels were found to be highest in spermatic vein and peripheral blood and low in testicular vein blood. The expected pattern of kisspeptin levels was not found in the testis fluids. It is concluded that kisspeptin is expressed in the monkey testis but its concentration does not show a specific pattern in different testicular fluids. Nevertheless, it is an important source of kisspeptin in peripheral circulation but its physiological significance is not known.

INTRODUCTION

Mammalian testicular development and its functional maintenance are mainly controlled by the pituitary gonadotrophins and testosterone. Many studies have now demonstrated the presence of many neuropeptides and their receptors in the testis, suggesting that these peptides are involved in the local regulation of testicular germ cell development and function. Testis is a complex organ which is functionally divided into the gamete and endocrine segments, where spermatogenesis and steroid hormone biosynthesis take place, respectively (Sharpe, 1994; de Kretser et al., 1998). The spermatogenic compartment is formed by the seminiferous epithelium, which is composed of Sertoli cells and various stages of germ cells. Sertoli cells create a blood testis barrier by maintaining the tight junctions between them which provide the seminiferous tubule an immunologically safe environment that is separated from the peripheral circulation. The endocrine compartment consists of interstitial cells (Leydig cells), the site of testosterone biosynthesis; macrophages, which are closely associated with Leydig cells and the lymphatic tissue area that surrounds this endocrine segment. The Leydig, peritubular, and Sertoli cells provide the necessary support for germ cell development and steroidogenesis (Damian, 1990). Testosterone functions as a male hormone but also regulates the maintenance of spermatogenesis (Sharpe, 1994; de Kretser et al., 1998). Its actions are aided by a variety of peptide hormones and growth factors. Many of these are locally produced by the testis and are involved in paracrine and autocrine processes (Damian, 1990). Many neuropeptides are expressed within testis including GnRH, CRH, vasoactive intestinal polypeptide (VIP), oxytocin, arginine vasopressin, TRH, somatostatin, opioid peptides and neuropeptide Y (NPY) (Li and Arimura, 2003). Spermatogenesis and steroidogenesis are dependent on normal stimulation by the pituitary gonadotrophins, FSH, and LH, whose release is stimulated by GnRH (Matsumoto et al., 1986). The pituitary gonadotrophins also

affect the expression of testicular neuropeptides via paracrine factors produced by Sertoli and Leydig cells (Li and Arimura, 2003).

Kisspeptin neuropeptides and their receptor, Kiss1r, are now well established in the central regulation of reproductive axis (Roseweir and Millar, 2009). Gonadal and testicular expression of Kiss1 and Kiss1r has been reported (Kotani et al., 2001). Our immunohistochemistry and RT-PCR data indicate considerable expression of kisspeptin in the adult monkey testis. Foregoing observations suggest a paracrine role of kisspeptin in the monkey testis. Accordingly, it can be postulated that kisspeptin may also be released from the monkey testis cells and can be detected in different testicular fluids and homogenates.

The present study was based on the particular idea that if kisspeptin is expressed by testicular cells then its concentration would be greater in the testicular tissue and a concentration gradient will most probably be present between testicular tissue, testicular vein, spermatic vein and peripheral blood circulation. Kisspeptin release was also measured from testicular tissues incubated in media to compare in vitro levels of kisspeptin with in vivo levels from testis. We expected to get a higher concentration of kisspeptin in the testis homogenate and then a decreasing trend in testicular, spermatic and peripheral veins.

MATERIALS AND METHODS

Animals

A total number of five adult intact male rhesus monkeys (*Macaca mulatta*), 7-9 years old, weighing 8.3 -14.7 kg was used in this study. Their testicular volumes were 19.1-61.4 ml. The animals were housed in individual cages and maintained under semi-controlled environmental conditions at temperature (25±3 °C) and light (06:00-18:00) in the Primate Facility of the Qauid-i-Azam University, Islamabad. Standard monkey diet supplemented with fresh fruits (09:00-09:30 h), boiled eggs (11:00 h) and bread (13:00-13:30 h) was provided daily. Water was available *ad libitum*. All animal experiments were approved in advance by the Departmental Committee for Care and Use of Laboratory Animals.

Collection of Testicular Vein, Spermatic Vein and Peripheral Blood from Adult Rhesus Monkey

The method for testicular vein (TV) and spermatic vein (SV) blood collection was adopted from that described by Waites et al. (1974). The animals were anesthetized with intramuscular injection of 10 mg/kg BW ketamine hydrochloride. Each testis was exposed and exteriorized aseptically through a 2-3 cm incision in the anterior aspect of the scrotum. For the collection of TV and SV blood, heparin coated 1ml insulin syringe fitted with 27 gauge needle was used. The needle was inserted in the testicular vein on the surface of the testis, and 1 ml blood was slowly collected from vein (Figure 1). The differentiation of the testicular vein from the artery was based on the fact that the veins run towards the proximal part of testis and form branches there, while artery runs around distal part of testis. Spermatic vein blood samples (1 ml) were also collected from one of the veins at the proximal end of the spermatic cord into heparinized tubes (Figure 1). Peripheral blood samples (PV) (2 ml) were collected from the saphenous vein in heparinized 3 ml syringes

from these monkeys. All the blood samples were taken during the time from 11:00 to 11:20 h. The samples were placed in tubes kept on ice. The blood samples were immediately centrifuged at 3000 rpm to extract plasma that was stored at -20 C^{o} until assay was done for kisspeptin.

Testicular Tissue Collection and Homogenate Preparation

For the preparation of testis homogenate, 100 mg of testis was collected from five intact adult rhesus monkeys through biopsy of left testis. The animals were anesthetized with intramuscular injection of 10 mg/kg BW ketamine hydrochloride. An incision was given in the anterior side of scrotum with a sharp scalpel blade. The testes were exteriorized from the scrotum. For testicular biopsy, a 2-3 cm incision was given in the tunica albugenia and vaginalis, at the proximal side of the testis, avoiding veins. A total of 100 mg of testicular tissue was taken carefully from the testis of each monkey. Tissue was weighed and immediately preserved in liquid nitrogen and then saved at -70°C. Afterwards, tunica vaginalis, peritonium and skin each were sutured separately. A preparation of penecillin and streptomycin was sprinkled on the wound to avoid infection. A single shot of long-acting antibiotic was given after surgery. Stitches were removed after 2 weeks.

The testis homogenate preparation was done as previously described by Laurenzana et al. (2002). For homogenate preparation, the testicular tissue was thawed and homogenized in 100 µl of 50 mM phosphate buffer (pH 7.4) using a pestle morter. Aliquots of the homogenates were stored at −80°C until kisspeptin assay was applied. For the measurement of kisspeptin in vitro culture medium from testicular fragments, medium was collected after 30 minutes of incubation of testicular tissue in that medium.

Testicular Tissue Incubation

Analysis of the direct effect of kisspeptin on testosterone and inhibin secretion was carried out by using the testicular slice incubation method which was done as previously described

by Roulet et al. (2006) with slight modifications. Testicular tissues biopsied from adult rhesus monkeys were de-capsulated, weighed and cut into five fragments of approximately 100 mg which were immediately transferred to fresh ice cold Dulbecco's modified Eagle's medium (DMEM) with PH 7.4. Testicular slices were incubated for 1 h in 2 ml DMEM in 5 ml culture tubes under 5% CO_2 and 95% air at 32 °C in a CO_2 incubator. After incubation for 1 h, the media was replaced with medium containing kisspeptin at five different dose rates (no dose, 1pm, 10pm, 100pm, 1000pm). Aliquots of 100 µl were then taken from each tube after 30, 60 and 120 min of incubation and stored at -20 °C until assay. Then, all media was removed and replaced with media containing 10IU HCG. 100 µl aliquots of the medium were taken after 3 h. Again all the media was replaced with medium containing 100 ng/ml FSH and 100 µl aliquots were taken after 2 h.

Statistical Analysis

Statistical comparisons for the mean plasma levels of kisspeptin were made by ANOVA, and paired t test. All data are presented as mean (± SEM). Results were considered statistically significant at $P < 0.05$. Statistical analyses were carried out using Graphpad Prism software version 6 (www.graphpadprism.org) (GraphPad Software. Inc, La Jolla, CA, USA).

RESULTS

Individual and mean concentration of kisspeptin like immunoreactivity (ir) in plasma from peripheral (saphenous vein) blood plasma, testicular vein and spermatic vein blood and testicular homogenate are shown in Table 3.1 and Figure 3.1. Measurable levels of kisspeptin like ir were found in all of the testicular fluids and peripheral plasma and the levels were all detectable over the range of standards used in the assay. Kisspeptin levels released in the medium of in vitro testicular fragments were also measured (Table 3.1 and Figure 3.1) and a statistically significant ($P < 0.05$) difference of kisspeptin like ir was

noted between in vitro and other fluids. The kisspeptin like ir was much less in in vitro sample as compared to in vivo testicular fluids (0.29 ± 0.08 vs. 12.20 ± 3.48 to 18.90 ± 4.21 ng/ml). The mean kisspeptin like ir in the testicular fluids and homogenate ranged from 12 to 18 ng/ml. The levels were quite comparable to the peripheral kisspeptin like ir. However, kisspeptin concentration was not significantly different between testicular venous plasma, spermatic vein plasma, testicular homogenate and peripheral plasma.

DISCUSSION

We previously described the Kiss1 protein and mRNA expression in the testicular tissue of rhesus monkey and therefore we hypothesized that if kisspeptin is being produced by the testis then kisspeptin levels would show a concentration gradient in different testicular fluids. We found quite fair and detectable levels of kisspeptin like ir in testicular homogenate preparation, testicular venous blood and testicular spermatic vein blood, but we did not find any statistically significant variations among these levels. We expected a decreasing trend of kisspeptin concentration from testicular homogenate to peripheral blood but such a pattern was absent. Kisspeptin like ir was noted to be higher in spermatic vein and next high in the peripheral blood, while, in the testis homogenate it was observed to be less than the peripheral blood. One reason for this might be the fidelity of the assay which we used for kisspeptin concentration measurement as this assay shows 100% cross reactivity with RFRP1 in addition to kisspeptins. However, assay validity for kisspeptin was evident by in range values of our samples with the positive quality control samples. kisspeptin levels in our samples were fairly in the range of positive controls for kisspeptin

Table 3.1. Comparison of individual and mean concentration of kisspeptin *in vitro* testicular incubation medium, peripheral blood plasma, spermatic vein plasma, testis vein plasma and homogenate of testicular tissue of adult rhesus monkey (n=5) under normal physiological conditions.

Animal	Kisspeptin levels (ng/ml)				
	Medium	Peripheral vein	Spermatic vein	Testis vein	Homogenate
M1	0.43	17.30	28.79	15.62	13.64
M2	0.51	5.80	14.43	17.79	20
M3	0.068	24.15	5.54	3.47	14.9
M4	0.29	10.85	26.59	19.93	5.24
M5	0.18	23.79	19.15	4.20	16.34
Mean	0.29±0.08	16.38±3.5	18.90±4.21	12.20±3.48	14.04±2.44

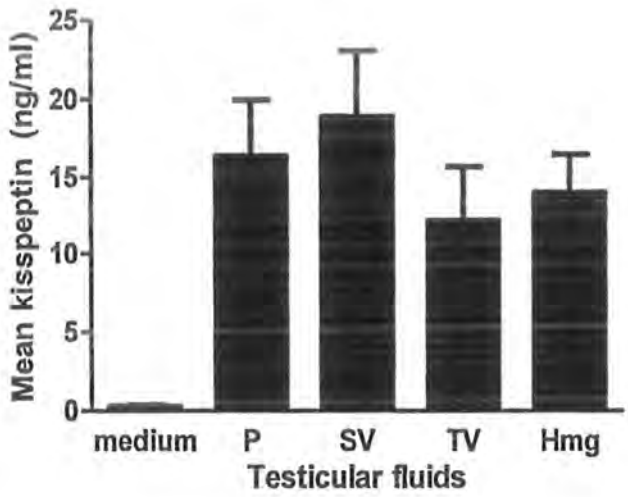

Figure 3.1. Comparison of kisspeptin like immunoreactivity in *in vitro* testicular incubation medium, peripheral blood plasma (PV), spermatic vein plasma (SV), testicular vein plasma (TV) and homogenate (Hmg) of testicular tissue of adult intact male rhesus monkey (n=5) under normal physiological conditions. One way ANOVA followed by post hoc test indicated that there was no significant variation of kisspeptin levels among the testicular fluids. However, kisspeptin levels in testicular fluids were significantly ($P< 0.05$) higher than that observed in in vitro testicular incubation medium.

which ranged from 0.4-0.6 ng/ml which indicated that specifically kisspeptin was measured. Interestingly, highest levels of kisspeptin like ir were observed in the spermatic vein. High kisspeptin levels in the spermatic vein blood may indicate some epididymal source. In the peripheral blood, kisspeptin levels were observed to be lower than the spermatic vein but higher than testicular vein and homogenate. The possible reason for this variance may be due to the fact that kisspeptin was added in the peripheral blood from different other peripheral tissues as well as from central axis. Although, kisspeptin levels were detectable in the homogenate and testicular vein but comparatively reduced kisspeptin levels may arise due to the effect of many other factors that may regulate the expression of kisspeptin in the testicular tissue. In the male, INSL3 is a major product of the testicular Leydig cells, it was also analyzed in different testicular fluid compartments and its concentration was shown to be the highest in the interstitial fluid and notable concentrations were found in the rete testis fluid. Further, a significant variation of its level was found in interstitial fluid and rete testis fluid (Anand-Ivell et al., 2009). In the case of kisspeptin, this variation was not observed. It is thus clear that the levels of different testicular factors are different in testicular compartments, and the trend in their levels is independent of each other, but certainly their variation between different testicular compartment or testicular fluids is effected by some other causes. Thus, variation of kisspeptin may be affected by the androgens or some other testicular factors. These may also affect the expression of kisspeptin in the testicular cells. What are these factors, it certainly needs further investigation. In the present study, the release of kisspeptin like ir was also noted from the in vitro incubated testicular tissue but no kisspeptin release was observed which substantiated the fact that in vivo other factors are also involved in regulating the release of kisspeptin from the testis. In summary, our data indicate that kisspeptin is present in testicular tissue of monkey but concentration difference is not

present between different testicular tissues. Nevertheless, this testicular kisspeptin is an important source of kisspeptin in peripheral circulation but its significance is not known.

General Discussion

GENERAL DISCUSSION

In the present research work we studied the role for Kiss1-Kiss1r signalling in testicular tissue of the adult male rhesus monkey by using three different approaches. Firstly we investigated the localization of the Kiss1 and Kiss1r proteins in the testicular tissue of the adult male rhesus monkey by using immunohistochemistry. *Kiss1* and *Kiss1r* mRNA expression was also investigated in the testis by using RT-PCR. In second part of this study we co-localized kisspeptin and Kiss1r with Sertoli cell specific marker inhibin and with germ cells specific marker T4 (TPX1). Thirdly, we assessed a direct effect of kisspeptin on testosterone and inhibin release from testicular tissue fragments in vitro. Lastly, we sought to find the levels of kisspeptin in different testicular fluids and testicular homogenate in order to assess a pattern in testicular release of kisspeptin in monkeys.

Our results indicated that Kiss1 and Kiss1r proteins were localized to the testicular tissue of the adult male rhesus monkey as shown by immunohistochemical data. Further, using double label immunohistochemistry, Kiss1 and Kiss1r proteins were localized to specific cell types within the seminiferous tubules. The double immunohistochemical analysis showed localization of both Kiss1 and Kiss1r in the germ cells and Kiss1r in Sertoli cells. By double labelling Kiss1 with 17βHSD and Kiss1r with 17βHSD, Kiss1 and Kiss1r expressions were notably absent in Leydig cells. Our immunohistochemical data were confirmed in our RT-PCR studies where a clear detectable mRNA expression of both *Kiss1* and *Kiss1r* was observed in the testicular tissue. These findings are in consistent with the reports which demonstrated the *Kiss1* and *Kiss1r* expression in the human testis (Kotani et al., 2001). Our data suggest an autocrine/paracrine role for Kiss1 in testicular tissue, with respect to testicular and spermatogenic function of the primate testis. Available evidences indicate that hypothalamus and pituitary are mainly involved in controlling the

spermatogenesis and steroidogenesis through LH and FSH (Leutjens et al., 2005). Nevertheless, an organized interaction among testicular cells and compartments has also been found to be involved in regulating the testicular functions. This local interaction may involve some physical support or some hormonal communication between the germ cells and Sertoli cells and between germ cells and germ cells (Weinbauer and Wessels, 1999; Weinbauer et al., 2010). It is reasonable to conclude that kisspeptin is produced by germ cells (spermatocytes/spermatids) and could act in paracrine manner on the Sertoli cells to modulate their functioning. It is also the possibility that germ cells may communicate with other germ cells through kisspeptin. Thus, kisspeptin produced in spermatocytes and spermatids could act on Sertoli cells and other types of germ cells in the adult monkey testis.

Available evidences have shown that both Sertoli cell and germ cells regulate the process of spermatogenesis in the seminiferous tubules. Sertoli cells provide a physical support to the developing germ cells as they produce blood testis barrier between them and in addition they produce several factors which have been found to be involved in regulating the spermatogenesis. Some of these factors which are produced by Sertoli cells include IGF-I, IGF-2, TGF-α, TGF β, basic FGF, interleukins (Skinner et al., 1991). Moreover, germ cells are also capable to modulate Sertoli cell activity (Huleihel and Lunenfeld, 2004). Androgen binding protein, plasminogen activator, and FSH-dependent cAMP production by Sertoli cells have been shown to be affected by germ cells (Skinner et al., 1992; Huleihel and Lunenfeld, 2002; Skinner et al., 2009). In continuation with this notion, our findings do provide a clear evidence that kisspeptin is another new factor through which germ cells can communicate with Sertoli cells. What could be the physiological significance of Kiss1-Kiss1r signalling within germ cells and between germ cells and Sertoli cells, this requires further investigation. Parenthetically, a functional involvement

of KISS1-KISS1R signalling in affecting the human spermatozoa motility has recently been indicated by Pinto et al. (2011).

In summary, present results based on double label immunohistochemical and RT-PCR data clearly demonstrated the presence of both Kiss1 and its receptor in the seminiferous tubules of the adult monkey testis. Both Kiss1 and Kiss1r were present in the germ cells while Kiss1r was also present on the Sertoli cells. Kiss1 and Kiss1r mRNA expression was also found in the testis. Both kisspeptin and its receptor are restricted to the seminiferous tubule which suggests that kisspeptin is possibly involved in the regulatory network of spermatogenesis.

The fact that kisspeptin and Kiss1r expressions are present in testicular tissue is evident from our data and findings of others (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Next, we tried to assess a direct action of kisspeptin on testicular functioning by using an in vitro direct effect of kisspeptin on testicular tissue. Therefore, in a second study, we tried to decipher a direct effect of kisspeptin on testicular functioning through the measurement of testosterone and inhibin levels in a culture of testicular tissue of adult male rhesus monkey. Although, we did not find any expression of Kiss1r in the Leydig cells but we still examined the possibility of an indirect effect of kisspeptin on testosterone because we hypothesized that kisspeptin may affect testosterone synthesis through some Sertoli cell produced factors which may affect the Leydig cell functioning. Kisspeptin was challenged to in vitro testicular tissue obtained from adult monkeys, at four different doses. However, kisspeptin exposure did not significantly increase the testosterone concentration at any dose while, hCG stimulation clearly elicited testosterone release indicating that our in vitro testicular tissue was competent. This result is in accordance with the result of our first experiment where we did not find any expression of Kiss1r protein in the Leydig cells

which are steroidogenic cells in the testis. Our finding rules out any indirect effect of kisspeptin mediated by Sertoli cell or germ cell on Leydig cells testosterone release in the monkey testis. A dose related effect of kisspeptin was absent in the testicular tissue culture, however, an independent effect of time was found. It is possible that some Sertoli cell released factor may have affected the testosterone synthesis in Leydig cells which caused the testosterone release after some time of incubation. Alternatively a more probable reason for the absence of dose effect may be that the dose administered was not very sufficient to affect and activate the testosterone synthesis. Use of comparatively increased doses may be able to produce a dose effect. It is pertinent to mention here, however, that in vitro effects of kisspeptin have been found to produce contradictory results where kisspeptin was administered to pituitary in vitro cultures. We conclude that kisspeptin at least within the doses used in the current study, does not affect the release of testosterone from the Leydig cells in the adult monkey testis.

However, interestingly, our results of lack of direct kisspeptin effect on monkey Leydig cells, are contrasted with different kisspeptin effect on the female gonads. Kiss1 and Kiss1r mRNAs were detected in adult rat, human and marmoset ovaries and kisspeptin-10 significantly increased the progesterone secretion in granulosa cells (Gaytan et al., 2009).

Since Kiss1r expression was evident on Sertoli cells in our immunohistochemistry studies, we also studied the effect of in vitro challenge of kisspeptin on inhibin secretion in the present study. We found that there was no dose effect of kisspeptin on inhibin release. Nevertheless, a significant inhibitory effect of time on the inhibin release was noticed suggesting an independent effect of time. FSH treatment to testicular tissue fragments in the culture caused a significant rise in inhibin, suggesting that the Sertoli cells were competent in our in vitro system. The most plausible reason for a lack of effect of

kisspeptin may be that kisspeptin in Sertoli cell have no role in inhibin biosynthesis or release. However, any other paracrine role of kisspeptin cannot be ruled out in Sertoli cells. It is also possible that kisspeptin only affects the stimulated inhibin release as suggested by the evidence that Kiss1 did not increase FSH release directly at the pituitary level, although it moderately enhanced GnRH-stimulated FSH secretion in vitro (Navarro et al., 2005). More recently, kisspeptin has been shown to enhance the hCG stimulated testosterone in adult rhesus monkeys (Irfan et al., 2008)

In summary, we did not find an effect of kisspeptin on testosterone and inhibin release from in vitro testicular tissue of adult male monkeys in the present study. Considering our immunohistochemistry and in vitro data, it can be concluded that although, Sertoli cells in monkey do express Kiss1r but this receptor activation is not relevant to endocrine function in terms of an indirect testosterone and direct inhibin release but may have a testicular action related to spermatogenesis.

We demonstrated the kisspeptin expression in the testicular tissue of rhesus monkey, therefore, we hypothesized that if kisspeptin is expressed within the testicular tissue then it should be released from the testis and there should be a gradient of kisspeptin levels in testis and testicular fluids. To assess the testicular release of kisspeptin in adult monkeys, we assayed levels of kisspeptin in various testicular fluids in the last part of our studies. We found well detectable levels of kisspeptin in testicular homogenate preparation, testicular venous blood and testicular spermatic vein blood. The assay validity was evident by the in range values of quality control samples. However, we did not find any significant variations among these levels. Kisspeptin levels were high in spermatic vein and peripheral blood. This suggests the possible kisspeptin expression in the epididymis and its release from the epididymis. Increased kisspeptin levels within the peripheral blood are most

probably due to the addition of kisspeptin from other peripheral tissues as well as from central axis. We speculate that comparatively reduced kisspeptin levels within testis homogenate are due to many testicular factors which act in a paracrine way to regulate the kisspeptin expression. We tend to conclude that monkey testis does not systematically release kisspeptin in the general circulation.

In summary, we found the expression of Kiss1 and Kiss1r in the adult male rhesus monkey testis particularly in Sertoli cells and germ cells. We did not note an effect of an in vitro kisspeptin challenge on testosterone and inhibin secretions and we were unable to find a concentration gradient of kisspeptin among different testicular fluids. Taking together, our data suggest a paracrine role of kisspeptin relevant to spermatogenesis in adult monkey testis which needs to be characterized in further studies. Kisspeptin may not be involved in hormonal regulation but it is quite possible that it is a part of regulatory network involved in primate spermatogenesis.

Kisspeptin is a neuropeptide and it is well established to be involved in modulating testicular functions through central regulation. Our observations in the present research work provide an idea of an important role of kisspeptin as a direct regulator of functions of the primate testis. The presence of kisspeptin receptor in testicular tissue also raises the possibility of kisspeptin being a player of paracrine interaction in testis. Kiss1 expression was observed in spermatocytes and spermatids and Kiss1r was observed in spermatocytes and Sertoli cells. This specific expression pattern of kisspeptin in tubular compartment suggests its role in spermatogenesis. Kisspeptin and its receptor expression were not observed in the Leydig cells. Lack of kisspeptin secretory activity of Leydig cells and Sertoli cells from in vitro testicular tissues suggests that it has no endocrine role. In

summery our results have broadened the understanding of kisspptin physiology relevant to reproductive system.

General Conclusions and Future Perspectives

In conclusion, our observations suggested that both kisspeptin and kisspeptin receptor are expressed within the testicular tissue of non-human primates. Kisspeptin has no endocrine effect on the testis but its paracrine effect cannot be ruled out. It is likely that a paracrine kisspeptin signalling mediates Sertoli cell - germ cell interaction in the monkey testis. Further, although, kisspeptin is present in the testis but it is not released in concentration gradient. Relatively high peripheral kisspeptin levels in adult monkeys are likely to be furnished by the testis among other tissues. Our data indicate the diversity of kisspeptin effect on HPG axis.

Complete elucidation of role of kisspeptin in the testis is needed to be explored in future studies. To find any other paracrine role of kisspeptin, it is needed to find the effect of kisspeptin on other testicular factors also, like INSL3 and GnIH. Because many testicular factors are expressed at specific spermatogenic stage, so it would be interesting to find the stage specific expression of kisspeptin to decipher a broad spermatogenic role. Expression of kisspeptin in the reproductive tissue makes an important segment of HPG axis circuitry. Elucidating the role of kissspeptin at this level would be helpful in resolving many issues related to HPG axis abnormalities and infertility in higher primates.

References

REFERENCES

Abbara A, Ratnasabapathy R, Jayasena CN, Dhillo WS 2013 The effects of kisspeptin on gonadotropin release in non-human mammals. Adv Exp Med Biol 784: 63-87.

Alastalo TP, Lonnstrom M, Leppa S, Kaarniranta K, Pelto Huikko M, Sistonen L, Parvinen M 1998 Stage-specific expression and cellular localization of the heat shock factor 2 isoforms in the rat seminiferous epithelium. Exp Cell Res 240: 16–27.

Aparicio SAJR 2005 Kisspeptins and GPR54—The new biology of the mammalian GnRH axis. Cell Metabol 1: 293-296.

Benahmed M 1997 Role of tumor necrosis factor in the male gonad. Contracept Fertil Sex 25: 569–571.

Berensztein E, Saraco N, Belgorosky A, Rivarola MA 2000 Secretion of inhibin B by human prepubertal testicular cells in culture. Eur J Endocrinol 142: 481-485.

Bergh A, Damber JE, Widmark A 1988 Hormonal control of testicular blood flow, microcirculation and vascular permeability. In: Cooke BA and Sharpe RM, eds. Molecular and Cellular Endocrinology of the Testis. New York: Raven Press;123-133.

Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C, Malli R, Sharabi A, Hiden U, Graier W, Knöfler M, Andreae F, Wagner O, Quaranta V, Desoye G 2004 Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. J Cell Sci 117:1319-1328.

Biran J, Ben-Dor S, Levavi-Sivan B 2008 Molecular identification and functional characterization of the kisspeptin/ kisspeptin receptor system in lower vertebrates. Biol Reprod 79: 776–786.

Bo-Abbas Y, Acierno Jr JS, Shagoury JK, Crowley Jr WF, Seminara SB 2003 Autosomal recessive idiopathic hypogonadotropic hypogonadism: Genetic analysis excludes mutations in the gonadotropin-releasing hormone (GnRH) and GnRH receptor genes. J Clin Endocrinol Metab 882: 730-2737.

Boepple PA, Hayes FJ, Dwyer AA, Raivio T, Lee H, Crowley WF Jr, Pitteloud N 2008 Relative roles of inhibin B and sex steroids in the negative feedback regulation of follicle stimulating hormone in men across the full spectrum of seminiferous epithelium function. J Clin Endocrinol Metab 93: 1809–1814.

Brailoiu GC, Dun SL, Ohsawa M, Yin D, Yang J, Chang JK, Brailoiu E, Dun NJ 2005 KiSS-1 expression and metastin-like immunoreactivity in the rat brain. J Comp Neurol 481: 314–329.

Brehm R, Zeiler M, Rüttinger C, Herde K, Kibschull M, Winterhager E, Willecke K, Guillou F, Lecureuil C, Steger K, Konrad L, Biermann K, Failing K, Bergmann M 2007 A sertoli cell-specific knockout of connexin43 prevents initiation of spermatogenesis. Am J Pathol 171: 19–31.

Bremner WJ, Matsumoto AM, Susman AM, Paulsen CA 1981 Follicle stimulating hormone and human spermatogenesis. J Clin Invest 68:1044-1052.

Brown RE, Imran SA, Ur E, Wilkinson M 2008 KiSS-1 mRNA in adipose tissue is regulated by sex hormones and food intake. Mol Cell Endocrinol 281:64–72.

Brown RE, Imran SA, Wilkinson M 2009 Ectopic brain peptides posing as adipokines: fat as a novel site of Kiss1 expression. In: Preedy VR, ed. International handbook of behavior, diet and nutrition. New York: Springer Publications.

Caraty A, Smith JT, Lomet D, Ben Said S, Morrissey A, Cognie J, Doughton B, Baril G, Briant C, Clarke IJ 2007 Kisspeptin synchronizes preovulatory surges in

cyclical ewes and causes ovulation in seasonally acyclic ewes. Endocrinology 148: 5258–5267.

Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, Dieguez C, Aguilar E, Sa´nchez-Criado JE, Pellicer A, Pinilla L, Gaytan F, Tena-Sempere M 2006 Expression of KiSS-1 in rat ovary: putative local regulator of ovulation. Endocrinology 147: 4852–4862.

Castellano JM, Navarro VM, Fernández-Fernández R, Nogueiras R, Tovar S, Roa J, Vazquez MJ, Vigo E, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M 2005 Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. Endocrinology 146: 3917-3925.

Chen Y, Yusenko MV, Kovacs G 2011 Lack of KISS1R expression is associated with rapid progression of conventional renal cell carcinomas. J Pathol 223: 46–53.

Clark RV 1976 Three dimensional organization of testicular interstitial tissue and lymphatic space in the rat. Anat Rec 184: 203-226.

Clarke IJ 2011 Control of GnRH secretion: One step back. Front. Neuroendocrinol 32: 367-375

Clarke IJ, Caraty A 2013 Kisspeptin and seasonality of reproduction. Adv Exp Med Biol 784:411-30.

Clarkson J and Herbison AE 2006 Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 147: 5817–5825.

Clarkson J, Hanb SK, Liua X, Leea K, Herbisona AE 2010 Neurobiological mechanisms underlying kisspeptin activation of gonadotropin-releasing hormone (GnRH) neurons at puberty. Mol Cell Endocrinol 324: 45-50.

Clements MK, McDonald TP, Wang R, Xie G, O'Dowd BF, George SR, Austin CP, Liu Q 2001 FMRF amide-related neuropeptides are agonists of the orphan G-protein-coupled receptor GPR54. Biochem. Biophys. Res. Commun 284: 1189–1193

Clermont Y 1972 Kinetics of spermatogenesis in mammals; seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52: 198-236.

Cockwell H, Wilkinson DA, Bouzayen R, Imran SA, Brown R, Wilkinson M 2012 KISS1 expression in human female adipose tissue. Arch Gynecol Obstet 287: 143-147.

Cohen PE, Pollard JW 1998 Normal sexual function in male mice lacking a functional type 1 interleukin-1 (Il-1) receptor. Endocrinology 139: 815–818.

Colledge WH 2008 Kisspeptins and GnRH neuronal signalling. Trends Endocrinol Metab 20: 115-121.

Colledge WH, Doran J, Mei H 2013 Model systems for studying kisspeptin signalling: mice and cells. Adv Exp Med Biol 784: 481-503.

Cunnah D, Perry L, Dacie JA, Grant DB, Lowe DG, Savage MO, Besser GM 1989 Bilateral testicular tumours in congenital adrenal hyperplasia: a continuing diagnostic and therapeutic dilemma. Clin Endocrinol 30:141–147.

Damian E 1990 The rat testis - a source of hypothalamic neuropeptide like factor. Endocrinology 28: 207–209.

d'Anglemont de Tassigny X, Fagg LA, Dixon JP, Day K, Leitch HG, Hendrick AG, Zahn D, Franceschini I, Caraty A, Carlton MB, Aparicio SA, Colledge WH 2007 Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc Natl Acad Sci USA 104: 10714-10719.

de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N 1998 Spermatogenesis. Hum Reprod 13: 1–8.

Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C 2008 Kisspeptin immunoreactive neurons in the equine hypothalamus interactions with GnRH neuronal system. J Chem Neuroanat 36:131–137.

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100: 10972–10976.

Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR 2005 Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab 90: 6609-6615.

Dufau M 1996 The luteinizing hormone receptor. In: A. H. Payne, M. P. Hardy, and L. D. Russell, eds. The Leydig Cell. Vienna, IL: Cache River Press; 333–350.

Dun SL, Brailoiu GC, Parsons A, Yang J, Zeng Q, Chen X, Chang JK, Dun NJ 2003 Metastin-like immunoreactivity in the rat medulla oblongata and spinal cord. Neurosci Lett 335: 197–201.

Eddie L W, Baker HWJ, Dulmanis A, Higginson RE, Hudson B 1978 Inhibin from cultures of rat seminiferous tubules. J Endocrinol 78:217-224.

Ehmcke J, Schlatt S 2006 A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction 132: 673–680.

Fawcett DW, Neaves WB, Flores MN 1973 Comparative observations on intertubular lymphatics and the organization of the interstitial tissue of the mammalian testis. Biol Reprod 9: 500-532.

Fijak M, Meinhardt A 2006 The testis immune privilege. Immunol Rev 213:66–81.

Filby AL, van Aerle R, Duitman J, Tyler CR 2008 The kisspeptin/gonadotropin-releasing hormone pathway and molecular signaling of puberty in fish. Biol Reprod 78:278–289.

Franca LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD 1998 Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59:1371–1377.

Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A 2006 Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. Neurosci Lett 401:225–230.

Franchimont P, Verstraelen-Proyard J, Hazee-Hagelstein MT, Demoulin RA, Bourtuignon JP, and Hustin J 1979 Inhibin: from concept to reality. Vitam Horm 37: 243-302.

Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL 2003 The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 312:1357-1363.

Gaytan F, Gaytan M, Castellano JM, Romero M, Roa J,. Aparicio IB, Garrido N, . Sanchez-Criado JE, Millar IRP, Pellicer A, Fraser HM, Tena-Sempere M 2009 KiSS-1 in the mammalian ovary: distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. Am J Physiol Endocrinol Metab 296: E520–E531.

Gaytan M, Castellano JM, Roa J, Sa´nchez-Criado JE, Tena-Sempere M, Gaytan F 2007 Expression of KiSS-1 in rat oviduct: possible involvement in prevention of ectopic implantation? Cell Tissue Res 329: 571–579.

George JT and Seminara SB 2012 Kisspeptin and the hypothalamic control of reproduction: lessons from the human endocrinology153: 5130–5136.

Goldberg SF, Miele ME, Hatta N, Takata N, Paquette-straub C, Freedman LP, Welch DR 2003 Melanome metstastasis suppression by chromosome 6: Evidence for a pathway regulated by CRSP3 and TXNIP. Cancer Res 63: 432-440

Gottsch ML, Clifton DK, Steiner RA 2009 From KISS1 to kisspeptins: An historical perspective and suggested nomenclature. Peptides 30: 4-9.

Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145: 4073–4077.

Greives TJ, Kriegsfeld LJ, Demas GE 2008 Exogenous kisspeptin does not alter photoperiod-induced gonadal regression in Siberian hamsters (Phodopus sungorus). Gen Comp Endocrinol 156:552–558.

Greives TJ, Mason AO, ScottiMA,Levine J, Ketterson ED, Kriegsfeld LJ, Demas GE 2007 Environmental control of kisspeptin: implications for seasonal reproduction. Endocrinology 148:1158–1166.

Groome NG. 1990 Monoclonal and polyclonal antibodies reactive with the 132 amino terminal sequence of the alpha subunit of human 32K inhibin. Hybridoma 9: 31-42.

Gutierrez-Pascual E, Martinez-Fuentes AJ, Pinilla L, Tena-Sempere M, Malagon MM, Castano JP 2007 Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinising hormone and growth hormone secretion. J Neuroendocrinol 19: 521–530.

Hall PF 1994 Testicular steroid synthesis: organization and regulation. In: Knobil. E and Neill. JD, eds. The Physiology of Reproduction. New York: Raven Press; 1335–1362.

Hameed S 2011 Kisspeptin and fertility. J Endocrinol 208: 97–105.

Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25: 11349-11356.

Haneji T, Koide SS, Tajima Y, Nishimune Y 1991 Differential effects of epidermal growth factor on the differentiation of type A spermatogonia in adult mouse cryptorchid testes in vitro. J Endocrinol 128:383–388.

Hara T, Tamura K, de Miguel MP, Mukouyama Y, Kim HJ, Kogo H, Donovan PJ, Miyajima A 1998 Distinct roles of oncostatin M and leukemia inhibitory factor in the development of primordial germ cells and Sertoli cells in mice. Dev Biol 201: 144–153.

Harrison RG and weiner JS 1949 Vascular patterns of the mammalian testis and their functional significance. J Exp Biol 26: 304-316.

Hauge-Evans AC, Richardson CC, Milne HM, Christie MR, Persaud SJ, Jones PM 2006 A role for kisspeptin in islet function. Diabetologia 49:2131–2135.

Haywood M, Spaliviero J, Jimemez M, King NJ, Handelsman DJ, Allan CM 2003 Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle stimulating hormone alone or in combination with testosterone. Endocrinology 144: 509–517.

Hedger MP 2002 Macrophages and the immune responsiveness of the testis. J Reprod Immunol 57: 19–34

Herbison AE 1998 Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocrin Rev 19: 302–330.

Hiden U, Bilban M, Kno¨ fler M, Desoye G 2007 Kisspeptins and the placenta: regulation of trophoblast invasion. Rev Endocr Metab Disord 8:31–39

Holstein AF, Maekawa M, Nagano T, Davidoff MS 1996 Myofibroblasts in the lamina propria of human seminiferous tubules are dynamic structures of heterogenous phenotype. Arch Histol Cytol 59: 109–125.

Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Kallo I 2010 The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. Eur J Neurosci 31: 1984–1998.

Hrabovszky E, Vida B, Horvath B, Keller E, Caraty A, Coen CW, Liposits Z, Kallo I, Distribution of kisspeptin-like immunoreactivity in the human hypothalamus: demonstration of neuronal contacts with type-I gonadotropin-releasing hormone neurons. Proc First World Conference on Kisspeptin Signaling in the Brain, Cordoba, Spain, 2008; p 73.

(http://www.informatics.jax.org/mgihome/nomen/gene.shtml)

Huleihel M, Lunenfeld E 2002 Involvement of intratesticular IL-1 system in the regulation of Sertoli cell functions. Mol Cell. Endocrinol 187: 125–132.

Huleihel M, Lunenfeld E 2004 Regulation of spermatogenesis by paracrine/autocrine testicular factors. Asian J Androl 6: 259-268

Ikeguchi M, Yamaguchi K and Kaibara N 2004 Clinical significance of the loss of KiSS-1 and orphan G-protein-coupled receptor (hOT7T175) gene expression in esophageal squamous cell carcinoma. Clin Cancer Res 10: 1379–1383.

Inoue N, Pheng V, Inamoto Y, Uenoyama Y, Tsukamura H, Ichikawa M, Maeda KI, Kisspeptin fibers directly contact with gonadotropin-releasing hormone fibers in the median eminence of female rats. Proc First World Conference on Kisspeptin Signaling in the Brain, Cordoba, Spain, 2008; p 74.

Inoue Y, Kamura T, Okuma A, Shigematsu T, Amanda S, Ogata R, Koga M, Nozaki M, Nakano H 1999 Efficacy of selective venous sampling to localise a small ovarian androgen producing tumor. J Obstet Gynecol Res 25: 231–236.

Irfan S, Anees M, Wahab F, Zaman U, Shahab M, Plant TM 2008 Evidence for a direct intratesticular action of kisspeptin in the adult rhesus monkey (*Macaca mulatta*). Program of the 90th Annual Meeting of the Endocrine Society, San Francisco, CA, USA, (abstract) OR52-6.

Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA 2004 Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80: 264–272.

Jacks F and Setchell BP 1973 A technique for studying the transfer of substances from venous to arterial blood in the spermatic cord of wallabies and rams. J Physiol 233: 17-18.

Jegou B 1993 The Sertoli-germ cell communication network in mammals. Int Rev Cytol 147:25–96.

Jegou B, Syed V, Sourdaine P, Byers S, Gerard N, Velez de la Calle J, Pineau C, Garnier DH, Bauche′ F 1992 The dialogue between late spermatids and Sertoli cells in vertebrates: a century of research. In: Nieschlag E, Habenicht UF. Eds. Spermatogenesis, fertilization, contraception molecular, cellular and endocrine events in male reproduction. Heidelberg: Springer; 57–97.

Jiang Y, Berk M, Singh LS, Tan H, Yin L, Powell CT, Xu Y 2005 KiSS1 suppresses metastasis of human ovarian cancer via inhibition of protein kinase C alpha. Clin Exp Metastasis 22: 369-376.

Kalamatianos T, Grimshaw SE, Poorun R, Hahn JD, Coen CW 2008 Fasting reduces KiSS-1 expression in the anteroventral periventricular nucleus (AVPV): effects of fasting on the expression of KiSS-1 and neuropeptide Y in the AVPV or arcuate nucleus of female rats. J Neuroendocrinol 20: 1089–1097.

Kanda S, Akazome Y, Matsunaga T, Yamamoto N, Yamada S, Tsukamura H, Maeda K, Oka Y 2008 Identification of KiSS-1 product kisspeptin and steroid-sensitive sexually dimorphic kisspeptin neurons in medaka (*Oryzias latipes*). Endocrinology 149: 2467–2476.

Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M 2007 Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 148: 1774–1783

Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E 2008 An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys *in vivo*. Endocrinology 149:4151–4157.

Kerr JB and De Kretser DM 1981 The cytology of the human testis. In: Burger H and De Kretser DM Eds. The Testis. New York: Raven Press; 141-169..

Kerr JB, Loveland KL, O'Bryan MK, de Kretser DM 2006 Cytology of the testis and intrinsic control mechanisms. In: Jimmy D. Neill Eds. Physiology of Reproduction. Elsevier Academic Press Publications.

Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K-I 2005 Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146(10): 4431-4436.

Knobil E 2005 Discovery of the hypothalamic gonadotropin-releasing hormone pulse generator and of its physiologic significance. Am J Obstet Gynecol 193: 1765–1766.

Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G, Parmentier M 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein coupled receptor GPR54. J Biol Chem 276: 34631–34636.

Lanfranco F, Gromoll J, Eckardstein SV, Herding EM, Neischlag E, Simoni M 2005 Role of sequence variations of the GnRH receptor and G-protein coupled receptor 54 gene in male idiopathic hypogonadotropic hypogonadism. Euro J Endocrinol 153:845-852.

Laurenzana EM., Balasubramanian G, Weis C, Blaydes B, Newbold RR, Delclos BK 2002 Effect of nonylphenol on serum testosterone levels and testicular steroidogenic enzyme activity in neonatal, pubertal, and adult rats. Chem Biol Interact 139: 23–41.

Le G F, and de Kretser DM 1982 Inhibin production by Sertoli cell cultures. Mol Cell Endocrinol 28:487-498.

Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-Nguyen AT, George SR, O'Dowd BF 1999 Discovery of a receptor related to the galanin receptors. FEBS Lett 446: 103–107.

Lee JH and Welch DR 1997 (a) Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display. Int J Cancer 71: 1035-1044.

Lee JH and Welch DR 1997 (b) Suppression of Metastasis in Human Breast Carcinoma MDA-MB-435 Cells after Transfection with the Metastasis Suppressor Gene, KiSS-1. Cancer Res 57: 2384-2387.

Lee JH, Miele ME, Hicks DJ, Phillips KK, JefferyM. Trent JM, Weissman BE, Welch DR 1996 KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst 88: 1731-1737

Lee YR, Tsunekawa K, Moon MJ, Um HN, Hwang JI, Osugi T, Otaki N, Sunakawa Y, Kim K, Vaudry H, Kwon HB, Seong JY, TsutsuiK 2009 Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. Endocrinology 150: 2837–2846.

Levalle O, Zylbersztein C, Aszpis S, Aquilano D, Terradas C, Colombani M, Aranda C, Scaglia H 1998 Recombinant human follicle-stimulating hormone administration increases testosterone production in men, possibly by a Sertoli cellsecreted nonsteroid factor. J Clin Endocrinol Metab 83: 3973–3976.

Li M and Arimura A 2003 Neuropeptides of the pituitary adenylate cyclase–activating polypeptide/vasoactive intestinal polypeptide/ growth hormone releasing hormone/secretin family in testis. Endocrine 20: 201–214.

Li Q, Roa A, Clarke IJ, Smith JT 2011 Seasonal variation in GnRH response to kisspeptin in sheep: possible kisspeptin regulation of the kisspeptin receptor. Presented at: 93rd Annual Meeting of the Endocrine Society, Boston, MA. June 4–7.

Li S, Ren J, Yang G, Guo Y, Huang L 2008 Characterization of the porcine kisspeptins receptor gene and evaluation as candidate for timing of puberty in sows. J Anim Breed Genet 125: 219–227.

Li S, Zhang Y, Liu Y, Huang X, Huang W, Lu D, Zhu P, Shi Y, Cheng CH, Liu X, Lin H 2009 Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (*Carassius auratus*). J Endocrinol 201: 407–418.

Lindstedt G, Nystrom E, Matthews C, Ernest I, Janson PO, Chatterjee K 1998 Follitropin (FSH) deficiency in an infertile male due to FSH beta gene mutation. A syndrome of normal puberty and virilization but underdeveloped testicles with azoospermia, low FSH but high lutropin and normal serum testosterone concentrations. Clin Chem Lab Med 36: 663–665.

Lofrano-Porto A, Casulari LA, Nascimento PP, Giacomini L, Naves LA, da Motta LD, Layman LC 2008 Effects of follicle stimulating hormone and human chorionic gonadotropin on gonadal steroidogenesis in two sibling with a follicle stimulating hormone beta subunit mutation. Fertil Steril 90: 1169-74.

Loveland KL, Schlatt S 1997 Stem cell factor and c-kit in the mammalian testis: lessons originating from mother nature's gene knockouts. J Endocrinol 153: 337–344.

Luetjens CM, Weinbauer GF, Wistuba 2005 Primate spermatogenesis: new insights into comparative testicular organisation, spermatogenic efficiency and endocrine control. J Biol Rev 80: 475-488.

Maddocks S and Setchell BP 1986 The composition of extracellular interstitial fluid collected with a push-pull cannula from the testes of adult rats. J Physiol 407: 363-372.

Maddocks S and Setchell BP 1989 Testosterone concentrations in testicular interstitial fluid collected with a push-pull cannula or by drip-collection from adult rats given testosterone or aminoglutethimide. J Endocrinol 121: 303-309.

Maddocks S and Sharpe RM 1989 Dynamics of testosterone secretion by the rat testis: implications for measurement of the intratesticular levels of testosterone. J Endocrinol 122: 323-329.

Maddocks S and Sharpe RM 1989 Interstitial fluid volume in the rat testis: androgen dependent regulation by the seminiferous tubules? J Endocrinol 120: 215-222.

Magee C, Foradori CD, Bruemmer JE, Arreguin-Arevalo JA, McCue PM, Handa RJ, Squires EL, Clay CM 2009 Biological and anatomical evidence for kisspeptin regulation of the hypothalamic-pituitary-gonadal axis of estrous horse mares. Endocrinology 150: 2813–2821.

Tena-Sempere M 2012 Deciphering puberty: novel partners, novel mechanisms. Eur J Endocrinol 167: 733–747.

Martinez de la Escalera G, Choi ALH, Weiner RI 1994 Biphasic GABAergic regulation of GnRH secretion in GT1 cells. Neuroendocrinology 59: 420–425.

McGuire NL, Bentley GE 2010 Neuropeptides in the gonads: from evolution to pharmacology. Frontier Pharmacol 1: 1-13.

Martinez-Chavez CC, Minghetti M,Migaud H 2008 GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia. Gen Comp Endocrinol 156: 224–233.

Mason AO, Greives TJ, Scotti MA, Levine J, Frommeyer S, Ketterson ED, Demas GE, Kriegsfeld LJ 2007 Suppression of kisspeptin expression and gonadotropic axis sensitivity following exposure to inhibitory day lengths in female Siberian hamsters. Horm Behav 52: 492–498.

Masui T, Doi R, Mori T, Toyoda E, Koizumi M, Kami K, Ito D, Peiper SC, Broach JR, Oishi S, Niida A, Fujii N, Imamura M 2004 Metastin and its variant forms suppress migration of pancreatic cancer cells. Biochem Biophys Res Commun

Mead EJ, Maguire JJ, Kuc RE, Davenport AP 2007 Kisspeptins are novel potent vasoconstrictors in humans, with a discrete localization of their receptor, G protein-coupled receptor 54, to atherosclerosis-prone vessels. Endocrinology 148: 140–147.

Mechaly AS, Vin˜ as J, Piferrer F 2009 Identification of two isoforms of the Kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (*Solea senegalensis*). Biol Reprod 80: 60–69.

Meinhardt A, O'Bryan MK, McFarlane JR, Loveland KL, Mallidis C, Foulds LM 1998 Localization of follistatin in the rat testis. J Reprod Fertil 112: 233-241.

Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA 2005 Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc Natl Acad Sci USA 102: 1761–1766

Miele ME, Robertson G, Lee JH, Coleman A, McGary CT, Fisher PB, Lugo TG and Welch DR 1996 Metastasis suppressed, but tumorogenicity and local invasiveness unaffected in the human melanoma cell line MelJuSo after introduction of human chromosome 1 or 6. Mol Carcinol 15: 284-299.

Mitchell DC, Stafford LJ, Li D, Bar-Eli M, Liu M 2007 Transcriptional regulation of KiSS-1 gene expression in metastatic melanoma by specificity protein-1 and its coactivator DRIP-130. Oncogene 26: 1739-47.

Mohamed JS, Benninghoff AD, Holt GJ, Khan IA 2007 Developmental expression of the Gprotein-coupled receptor 54 and three GnRH mRNAs in the teleost fish cobia. J Mol Endocrinol 38: 235–244.

Moon JS, Lee YR, Oh da Y, Hwang JI, Lee JY, Kim JI, Vaudry H, Kwon HB, Seong JY 2009 Molecular cloning of the bullfrog kisspeptin receptor GPR54 with high sensitivity to *Xenopus* kisspeptin. Peptides 30: 171–179.

Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekeres PG, Sarau HM, Chambers JK, Murdock P, Steplewski K, Shabon U,

Miller JE, Middleton SE, Darker JG, Larminie CG, Wilson S, Bergsma DJ, Emson P, Faull R, Philpott KL, Harrison DC 2001 AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem 276 28969–28975.

Murphy KG 2005 Kisspeptins: regulators of metastasis and the hypothalamic-pituitary-gonadal axis. J Neuroendocrinol 17: 519-525.

Nakamura Y, Aoki S, Xing Y, Sasano H, Rainey WE 2007 Metastin stimulates aldosterone synthesis in human adrenal cells. Reprod Sci 14: 836–845.

Nanjappa MK, Simon L, Akingbemi BT 2012 The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. Biol Reprod 135: 1-35.

Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M 2004 Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145: 4565–4574.

Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M 2005 a Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology 146: 156–163.

Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M 2005 b Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle stimulating hormone secretion in the rat. Endocrinology 146:1689–1697

Nocillado JN, Levavi-Sivan B, Carrick F, Elizur A 2007 Temporal expression of G-protein-coupled receptor 54 (GPR54), gonadotropin-releasing hormones (GnRH), and dopamine receptor D2 (drd2) in pubertal female grey mullet, *Mugil cephalus*. Gen Comp Endocrinol 150: 278–287.

Oakley AE, Clifton DK, Steiner RA 2009 Kisspeptin signaling in the brain. Endocr Rev 30: 713-743.

O'Bryan MK, Sebire K, Meinhardt A, Edgar K, Keah HH, Hearn MT, De Kretser DM 2001 Tpx-1 is a component of the outer dense fibers and acrosome of rat spermatozoa. Mol Reprod Dev 58: 116-25.

Ogawa K, Hasshimoto O, Kurohmaru M, Mizutani T, Sugino H, Hayashi Y., Folllistatin like immunoreactivitiy in the cytoplasm and nucleus of spermatogenic cells in the rats. Euro J Endocrinol 1997 137: 523-529.

Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda KI, Okamura H 2009 Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. J Neuroendocrinol 21: 813–821.

Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M 2001 Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411: 613–617.

Parhar IS, Ogawa S, Sakuma Y2004 Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. Endocrinology 145: 3613–3618.

Patterson M, Murphy KG, Thompson EL, Patel S, Ghatei MA, Bloom SR 2006 Administration of kisspeptin-54 into discrete regions of the hypothalamus potently

increases plasma luteinising hormone and testosterone in male adult rats. J Neuroendocrinol 18: 349-354.

Phillip M, Arbelle JE, Segev Y, Parvari R 1998 Male hypogonadism due to a mutation in the gene for the beta-subunit of follicle stimulating hormone. N Engl J Med 338: 1729–1732.

Phillips BT, Gassei K, Orwig KE 2010 Spermatogonial stem cell regulation and spermatogenesis. Phil Trans R Soc B 365: 1663-1678.

Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M 2012 Kisspeptins and reproduction: physiological roles and regulatory mechanisms. Physiol Rev 92:1235-316.

Pinto FM, Cejudo-Roman A, Ravina CG, Fernandez-Sanchez M, Martın-Lozano D, Illanes M, Tena-Sempere M, Candenas ML 2011 Characterization of the kisspeptin system in human spermatozoa. Int J Androl 35: 63–73.

Plant TM 2006 The role of KiSS-1 in the regulation of puberty in higher primates. Eur J Endocrinol 155: 511-16.

Plant TM 2008 Hypothalamic control of the pituitary-gonadal axis in higher primates: key advances over the last two decades. J Neuroendocrinol 20: 719-726.

Plant TM, Ramaswamy S, Dipietro M 2006 Repetitive activation of hypothalamic G protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (*Macaca mulatta*) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology 147: 1007-1013.

Plant TM, Ramaswamy S, Simorangkir D, Marshall GR 2005 Postnatal and pubertal development of the rhesus monkey (*Macaca mulatta*) testis. Ann NY Acad Sci 1061: 149–162.

Pompolo S, Pereira A, Estrada KM, Clarke IJ 2006 Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. Endocrinology 147:804–810.

Popa SM, Clifton DK, Steiner RA 2008 The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction Annu Rev Physiol 70: 213–38.

Ramaswamy S, Dwarki K, Ali B, Gibbs RB, Plant TM 2013 The decline in pulsatile GnRH release, as reflected by circulating LH concentrations, during the infant juvenile transition in the agonadal male rhesus monkey (*Macaca mulatta*) is associated with a reduction in kisspeptin content of KNDy neurons of the arcuate nucleus in the hypothalamus. Endocrinology 154: 1845-1853.

Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM 2008 Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (*Macaca mulatta*) as revealed by double immunofluorescence and confocal microscopy. Endocrinology 149: 4387–4395

Revel FG, Saboureau M, Masson-Pe´vet M, Pe´vet P, Mikkelsen JD, Simonneaux V 2006 Kisspeptin mediates the photoperiodic control of reproduction in hamsters. Curr Biol 16:1730–1735.

Richard N, Galmiche G, Corvaisier S, Caraty A, Kottler ML 2008 KiSS-1 and GPR54 genes are co-expressed in rat gonadotrophs and differentially regulated in vivo by oestradiol and gonadotrophin-releasing hormone. J Neuroendocrinol 20:381–393.

Richter-Unruh A, Jorch N, Wessels HT, Weber EA, Hauffa BP 2002 Venous sampling can be crucial in identifying the testicular origin of idiopathic male luteinising hormone independent sexual precocity. Eur J Pediatr 161:668–671.

Roa J, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M 2008 New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Front Neuroendocrinol 29: 48-69.

Romano F, Tripiciano A, Muciaccia B, De Cesaris P, Ziparo E, Palombi F, Filippini A 2005 The contractile phenotype of peritubular smooth muscle cells is locally controlled: Possible implications in male fertility. Contraception 72: 294–297.

Roseweir AK and Millar RP 2009 The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update 15: 203–212.

Roulet V, Denis H, Staub C, Le Tortorec A, Delaleu B, Satie A P, Patard J J, Jégou B, Dejucq-Rainsford N 2006 Human testis in organotypic culture: application for basic or clinical research. Hum Reprod 21: 1564–1575.

Rozen S and Skaletsky HJ 2000 Primer3 on the www for general users and for biologist programmers. In: Krawetz S, Misener S, Eds. Bioinformatics Methods and Protocols: Methods in molecular Biology. Totowa, NJ: Humana Press; 365-386.

Russel LD, Ettlin RA, Sinha Hikim AP, Clegg ED 1990 Histological and histopathological evaluation of the testis. Cache River Press Clearwater Florida.

Sanchez-Carbayo M, Capodieci P, Cordon-Cardo C 2003 Tumor suppressor role of KiSS-1 in bladder cancer: loss of KiSS-1 expression is associated with bladder cancer progression and clinical outcome. Am J Pathol 162: 609-617.

Schlatt S, Arslan M, Weinbauer GF, Behre HM, Nieschlag E 1995 Endocrine control of testicular somatic and premeiotic germ cell development in the immature testis of the primate *Macaca mulatta*. Eur J Endocrinol 133: 235–247.

Schlatt S, Weinbauer GF, Arslan M, Nieschlag E 1993 Appearance of a smooth muscle actin in peritubular cells of monkey testes is induced by androgens, modulated by follicle-stimulating hormone, and maintained after hormonal withdrawal. J Androl 14:340–350.

Seamans MC, Roser JF, Linford RL, Liu IK, Hughes JP 1991 Gonadotropin and steroid concentrations in jugular and testicular venous plasma in stallions before and after GnRH Injection. J Reprod Fertil 44: 57–67.

Seminara SB and Kaiser UB 2005 New gatekeepers of reproduction: GPR54 and its cognate ligand, KiSS-1. Endocrinology 146:1686–1688.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JrJ, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley JrWF, Aparicio SA, Colledge WH 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349: 1614–1627.

Setchell BP 1978 The mammalian testis. Elek Books, London, and Cornell University Press, Ithaca.

Setchell BP and Galil KAA 1983 Limitations imposed by testicular blood flow on the function of the Leydig cells in vivo. Aust J Biol Sci 36: 285-293.

Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, PlantTM 2005 Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci USA 102: 2129–2134.

Sharpe RM 1983 Local control of testicular function. Q J Exp Physiol 68: 265-287

Sharpe RM 1984 Intratesticular factors controlling testicular function. Biol. reproduction 30: 29-49.

Sharpe RM 1994 Regulation of spermatogenesis. In: Knobil, E. and Neill, J. D, Eds. The physiology of reproduction. New York: Raven Press.

Sharpe RM, McKinnell C, McLaren T, Millar M, West TP, Maguire S, Gaughan J, Syed V, Je'gou B, Kerr JB, Saunders PTK 1994 Interactions between androgens, Sertoli cells and germ cells in the control of spermatogenesis. In: Verhoeven G,

Habenicht UF, eds. Molecular, cellular endocrinology of the testis. Heidelberg: Springer; 115–143.

Shibata M, Friedman RL, Ramaswamy S, Plant TM 2007 Evidence that down regulation of hypothalamic KiSS-1 expression is involved in the negative feedback action of testosterone to regulate luteinising hormone secretion in the adult male rhesus monkey (*Macaca mulatta*). J Neuroendocrinol 19: 432–438.

Skinner KM 1992 Growth factors in gonadal development. J Animal Sci 30-41.

Skinner KM, Nilsson EE, Bhandari KR 2009 Cell – Cell Signaling in the Testis and Ovary, In: Edward AD, Ralph AB, eds. Handbook of Cell Signaling, 2nd ed., Academic Press.

Skinner MK & MD Griswold 1980 Sertoli cells synthesize and secrete a transferrin-like protein. J Biol Chem. 255: 9523-9525.

Skinner MK 1991 Cell–cell interactions in the testis. Endocr Rev 12: 45–77.

Smith JT 2009 Sex steroid control of hypothalamic Kiss1 expression in sheep and rodents: comparative aspects. Peptides 30: 94-102.

Smith JT, Clay CM, Caraty A, Clarke IJ 2007 KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology 148:1150–1157

Smith JT, Clifton DK, Steiner RA 2006 Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction 131: 623-630.

Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, Bateman K, Goodman RL, Tilbrook AJ,UbukaT, Bentley GE, Clarke IJ, Lehman MN 2008 Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology 149: 5770–5782.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA 2005a Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146: 3686-3692.

Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA 2005b Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146: 2976–2984.

Smith JT, Li Q, Yap KS, Shahab M, Roseweir AK, Millar RP, Clarke IJ 2011 Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence. Endocrinology 152: 1001–1012.

Smith JT, Pereira A, Rao A, Clarke IJ 2006 Kisspeptin-10 stimulates luteinising hormone release from the ovine pituitary gland in vitro. Front Neuroendocrinol 27: 63–79.

Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ 2008 Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge: evidence that gonadotropes are not direct targets of kisspeptin *in vivo*. Endocrinology 149: 1951–1959.

Smith JT, Saleh SN, Clarke IJ 2009 Seasonal and cyclical change in the luteinizing hormone response to kisspeptin in the ewe. Neuroendocrinology 90: 283–91.

Sridharan S, Simon L, Meling DD, Cyr DG, Gutstein DE, Fishman GI, Guillou F, Cooke PS 2007 Proliferation of adult Sertoli cells following conditional knockout of the gap junctional protein GJA1 (connexin 43) in mice. Biol Reprod 76: 804–812.

Steinberger A, and Steinberger E 1976 Secretion of an FSH inhibiting factor by cultured Sertoli cells. Endocrinology 99: 918-921.

Suzuki S, Kadokawa H, Hashizume T 2008 Direct kisspeptin-10 stimulation on luteinizing hormone secretion from bovine and porcine anterior pituitary cells. Anim Reprod Sci 103: 360–365.

Takaimya K, Yamamoto A, Furukawa K, Zhao J, Fukumoto S, Yamashiro S, Okada M, Haraguchi M, Shin M, Kishikawa M, Shiku H, Aizawa S, Furukawa K 1998 Complex gangliosides are essential in spermatogenesis of mice: possible roles in the transport of testosterone. Proc Natl Acad Sci USA 95: 12147–12152.

Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC 2008 A GPR54-activating mutation in a patient with central precocious puberty. N Engl J Med 358: 709-715.

Tena-Sempere M 2006 GPR54 and kisspeptin in reproduction. Hum ReprodUpdate 12: 631-639.

Tena-Sempere M and Huhtaniemi I 2003 Gonadotropins and gonadotropin receptors. In: Fauser BCJM Ed. Reproductive Medicine, Molecular, Cellular and Genetic Fundamentals. New York: Parthenon Publishing; 225–244.

Terada N, Ohno N, Saitoh S, Saitoh Y, Komada M, Kubota H 2010 Involvement of a membrane skeletal protein, 4.1G, for Sertoli/germ cell interaction. Reproduction 139: 883–892.

Teraoa Y, Kumanob S, Takatsub Y, Hattoric M, Nishimurac A, Ohtakib T, Shintani Y 2004 Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. Biochimica et Biophysica Acta 1678: 102– 110

Terasawa E and Fernandez DL 2001 Neurobiological mechanisms of the onset of puberty in primates. Endocr Rev 22: 111-151.

Thompson EL, Murphy KG, Patterson M, Bewick GA, Stamp GWA, Curtis AE, Cooke JH, Jethwa PH, Todd JF, Ghatei MA, Bloom SR 2006 Chronic

subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. Am J Physiol Endocrinol Metab 291: 1074–1082.

Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillo WS, Todd JF, Ghatei MA, Bloom SR 2004 Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. J Neuroendocrinol 16: 850–858.

Tilbrook AJ, de Kretser DM, Clarke IJ 1992 A role for inhibin in the regulation of the secretion of follicle stimulating hormone in male domestic species. Dom Anim Endocrinol 9: 243–260

Tobari Y, Iijima N, Matsumoto K, Okanoya K, Ozawa H 2008 Distribution of kisspeptin like immunoreactivity in the brain of the bird, *Taeniopygia guttata.* Proc First World Conference on Kisspeptin Signaling in the Brain, Cordoba, Spain, p 115 96.

Tovar S, Vázquez MJ, Navarro VM, Fernandez-Fernandez R, Castellano JM, Vigo E, Roa J, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M 2006 Effects of single or repeated intravenous administration of kisspeptin upon dynamic LH secretion in conscious male rat. Endocrinology 147: 2696–2704.

Verhoeven G, and Franchimont P 1983 Regulation of inhibin secretion by Sertoli cell enriched cultures. Acta Endocrinol 102: 136-143.

Vliegen M, Schlatt S, Weinbauer GF, Bergmann M, Groome NP, Nieschlag E 1993 Localization of inhibin/activin subunits in the testis of adult nonhuman primates and men. Cell Tissue Res 273: 261–268.

Wahab F, Bano R, Jabeen S, Irfan S, Shahab M 2010 Effect of peripheral kisspeptin administration on adiponectin, leptin, and resistin secretion under fed and fasting condition in the adult male rhesus monkey (*Macaca mulatta*). Horm Metab Res 42: 570-4.

Wahab F, Ullah F, Chan YM, Seminara SB, Shahab M 2011 Decrease in hypothalamic *Kiss1* and *Kiss1r* expression: a potential mechanism for fasting-induced suppression of the HPG axis in the adult male rhesus monkey (*Macaca mulatta*) Horm Metab Res: 81–85.

Waites GMH and Einer JN 1974 Collection and analysis of rete testis fluid from macaque monkeys. J Reprod Fert 41: 505-508.

Waites GMH and Gladwell RT 1982 Physiological significance of fluid secretion in the testis and blood-testis barrier. Physiol Rev 62: 624-671.

Waites GMH and Moule GR 1961 Relation of vascular heat exchange to temperature regulation in the testis of the ram. J Reprod Fertil 2: 213-224.

Weinbauer GF, Luetjens CM, Simoni M, Nieschlag E 2010 Physiology of testicular function. In Nieschlag E, Eds. Andrology. Berlin Heidelberg: Springer-Verlag.

Weinbauer GF and Wessels J 1999 Paracrine' control of spermatogenesis. andrologia 31: 249–262.

Weinbauer GF, Niehaus M, Nieschlag E 2004 The role of testosterone in spermatogenesis. In: Nieschlag E, Behre HM, eds. Testosterone: Action, deficiency, substitution. Cambridge: Cambridge University Press; 173–206.

Weinbauer GF, Schlatt S, Walter V, Nieschlag E 2001 Testosterone-induced inhibition of spermatogenesis is more closely related to suppression of FSH than to testicular androgen levels in the cynomolgus monkey (*Macaca fascicularis*). J Endocrinol 168: 25–38.

Weinbauer GF, Wessels J 1999 Paracrine control of spermatogenesis. Andrologia 31:249–262.

West A, Vojta PJ, Welch DR, Weissman BE 1998 Chromosome localization and genomic structure of the KiSS-1 metastasis suppressor gene (KISS1).Genomics 54:145-148

Xavier d'Anglemont de Tassigny X and Colledge WH 2010 The role of kisspeptin signaling in reproduction. Physiology 25: 207-217.

Xiaoa Y, Nia Y, Huanga Y, Wua J, Grossmannb R, Zhao R 2011 Effects of kisspeptin-10 on progesterone secretion in cultured chicken ovarian granulosa cells from preovulatory (F1–F3) follicles. Peptides 32: 2091–2097.

APPENDIX 1

1. Tariq AR, Shahab M, Clarke IJ, Pereira A, Smith JT, Khan SH, Sultan J, Javed S, Anwar T 2013 Kiss1 and Kiss1 receptor expression in the rhesus monkey testis: a possible local regulator of testicular function. Cent Eur J Biol 8: 968-974.

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Kiss1 and Kiss1 receptor expression in the rhesus monkey testis: a possible local regulator of testicular function

Research Article

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Abstract: Background: Kisspeptin, a *KISS1* gene product, stimulates GnRH neurons in the hypothalamus, but some recent studies have also suggested a direct effect on gonads. We aimed to localise Kiss and Kiss1 receptor (Kiss1r) in adult rhesus monkey testis. Experimental Procedures: Expression of Kiss1 and Kiss1r was detected in testicular tissue of rhesus monkey using immunohistochemistry and reverse transcriptase PCR (RT-PCR). Dual immunohistochemistry was used to colocalize Kiss1 with germ cell marker T4 and Kiss1r with Inhibin (Sertoli cell marker) using specific antibodies for all. Immunohistochemistry of testis was conducted on formaldehyde fixed tissues using 5μ thick sections. Using gene specific primers, RT-PCR was carried out to find expression of Kiss1 and *Kiss1r* mRNAs in testis. Results: Kiss1 immunoreactivity was localised to spermatocytes and spermatids and Kiss1r was observed in spermatocytes and Sertoli cells. Double-label immunohistochemistry co-localized Kiss1 and T4 in spermatids and Kiss1r and Inhibin were co-localized in Sertoli cells. RT-PCR showed mRNA expression of *Kiss1* and *Kiss1r* in adult rhesus monkey testis. Conclusions: Present results indicate for the first time the presence of Kiss1 and Kiss1r in adult primate testis. These suggest a possible autocrine/paracrine role of kisspeptin in non-human primate testis. Kiss1-Kiss1r in testis suggests possible direct involvement in the regulatory network involved in spermatogenesis.

Keywords: *Immunohistochemistry • Spermatogenesis • Germ cells • Sertoli cells*

1. Introduction

Kisspeptins (Kiss1) are encoded by the *KISS1* gene and act through GPR54 (now termed Kiss1r) [reviewed in 1]. The gene was originally discovered as a metastasis-suppressor in 1996 [2] and the gene product is a 145-amino acid peptide, from which is cleaved a 54-amino acid protein known as kisspeptin-54 [3], Kiss1r was initially described in the rat in 1999 [4] and shortly thereafter, the human homolog of GPR54 (KISS1R) was identified [5]. Kiss1 are products of the *KISS1* gene and are RF-amide (Arg-Phe-NH2) peptides that were described in 2001 as ligands for Kiss1r [5-7].

Kiss1 neurons are found in the arcuate nucleus and the anteroventral preoptic nucleus in the brain [8,9] and the Kiss1 peptides play a significant role in initiating puberty and reproductive function. Two research groups working independently in 2003 described a pivotal role of Kiss1-Kissr signaling in reproduction specifically that mutations in *KISS1R* were associated with the idiopathic hypothalamic hypogonadism and impaired pubertal maturation found in their patients [10,11]. Studies in a wide range of species now show that Kiss1 stimulates gonadotropin secretion [reviewed in 12]. Kisspeptin-10 is the shortest form of Kiss1 and elicits secretion of GnRH as measured in the CSF of sheep [13] and in hypophysial portal blood [14]. Consistent with its role within the brain, the *Kiss1r* is expressed by GnRH neurons [13,15,16].

Peripheral expression of Kiss1 and Kiss1r has also been observed in several tissues. The human placenta expresses *KISS1* and *KISS1R* and lower levels of

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expression have been reported in the human testis and small intestine [5]. *KISS1R* gene expression has also been noted in the pancreas, pituitary gland and spinal cord [7].

Castellano *et al.* [17] reported expression of *Kiss1* and *Kiss1r* in the rat ovary throughout the estrous cycle. Moderate expression of *KISS1r* expression has also been demonstrated in the human testis [5,6]. The presence of KISS1 and its receptor have been recently demonstrated in mature human spermatozoa and small but significant changes in sperm motility were induced by KISS1 and reduced in the presence of the KISS1 receptor antagonist p234 [18]. Thus, it was proposed that KISS1, acting *via* KISS1r could activate signal transduction pathways to modulate sperm movement [18]. Further, during a study of continuous intravenous administration of human metastin 45-54 on HPT axis in male monkeys, it was observed that for a given LH concentration, testosterone levels were invariably higher in response to metastin infusion [19]. Foregoing evidence raises a possibility that there is some intra-testicular action of kisspeptin which is responsible for amplified testosterone production. The present study was, therefore, designed to examine Kiss1 and Kiss1r protein and mRNA to particular cell types in the testes of rhesus monkeys in order to understand a possible direct testicular action of Kiss1-Kiss1r signaling.

2. Experimental Procedures

2.1 Animals

Eleven adult intact male rhesus monkeys (*Macaca mulatta*), 7-9 years old, weighing 8.3-14.7 kg were housed in individual cages and maintained under controlled environmental conditions in the Primate Facility of the Qauid-i-Azam University. The experiments were carried out during March and April. Standard monkey diet supplemented with fresh fruits, boiled eggs and water was provided daily. All animal experiments were approved in advance by the Departmental Committee for Care and Use of Laboratory Animals.

2.2 Tissue collection

For immunocytochemical studies, 6 adult rhesus monkeys were administered a lethal dose of ketamine hydrochloride (Ketler, Astarapin, Germany) at a dose of 10 mg/kg (i.m.) followed by a 25-30 mg/ml BW intravenous injection. Testicular tissues (100 mg) were obtained from one testis of each animal. For measures of RNA, by RT-PCR, tissues were collected from a further 5 rhesus monkeys by unilateral biopsy under sterile surgical conditions following anesthesia with ketamine hydrochloride (15 mg/kg). These tissues 1000 mg were snap frozen in liquid nitrogen and kept at -70°C until used for RNA extraction.

2.3 Tissue fixation and processing

For immunocytochemistry, testicular tissues were immersion-fixed in formaldehyde-alcohol-acetic acid fixative (60 ml absolute alcohol (Sigma-Aldrich Laborchemikalien GmbH, Steinheim, Germany), 30 ml of formaldehyde (Merck, Darmstadt, Germany) and 10 ml of glacial acetic acid (Merck) for 4-5 hours. The tissues were then passed through graded alcohols and placed in cedar wood oil (Serva Electophoresis GmBH, Heidelberg, Germany) until they appeared clear. Tissues were then washed twice (10 min) with benzol (Sigma-Aldrich), placed in benzol/paraplast (Fluka AG Buchs, Switzerland) (1:1) for 20 min and then washed 3 times (3x12 h) in Paraplast (Sakura, Tokyo, Japan) at 60°C to keep it in a liquid state. Finally, the tissues were embedded in paraffin and sectioned at 5 µm on a Shandon Finesse 325 microtome (Thermo Electron Cooperation, Waltham, MA, USA).

2.4 Fluorescence immunocytochemistry

2.4.1 Antibodies

We used a kisspeptin antibody raised in sheep against synthetic human kisspeptin-54 (GQ2) kindly provided by Prof Dr. Stephen Bloom (Imperial College, London, UK), at a dilution of 1:2000). Rabbit anti-AXOR12 (Kiss1r) (Phoenix Pharmaceuticals, Brisbane, Australia) was used at a dilution of 1:1000 to localize the Kiss1r. Donkey anti-sheep Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 488 (Life Technologies, Melbourne, Australia) were used as secondary antibodies at a dilution 1:500 for detection of immunostaining.

For identification of Sertoli cell, Leydig cells and sperm following antibodies and dilutions were used: mouse monoclonal anti human inhibin alpha(R1) (Sertoli cell marker, diluted 1:100; Serotec, Melbourne, Australia) Goat anti-17β-HSD3 (c-14) (Leydig cell marker, diluted 1:100; Santa Cruz Biotechnologies, Victoria, Australia). Rabbit anti T4 was used as a sperm marker antibody (diluted 1:200) and was provided by Moira O'Bryan (Monash Institute of Reproduction and Development, Monash Medical Centre, Clayton, Australia). The following fluorescent labeled secondary antibodies (Molecular Probes Inc Eugene, Oregon, USA) were used: Goat anti-mouse Alexa 546 (diluted 1:500) for detection of inhibin staining, streptavidin texas red (diluted 1:1000) for 17βHSD3 staining and goat anti-rabbit Alexa 546 (diluted 1:500) for T4 staining. Cell nuclei were stained with a DAPI nuclear stain (Life Technologies, Melbourne, Australia). Omission

of primary antibody served as a negative control in all experiments.

2.4.2 Immunohistochemistry procedure

Paraffin sections were dewaxed in xylene, rehydrated through descending grades of alcohol and rinsed in distilled water. The sections were then washed in 0.1 M Tris-buffered saline (TBS) (pH 7.4), mounted on Superfrost slides and dried over-night. Antigen retrieval was then performed using citrate buffer (pH 6.0) and heating in a microwave oven (2x3 min). The slides were then washed in TBS (3x10 min) and incubated in 10% normal goat serum/0.3% Triton X-100 in TBS for 2 h at room temperature. Sections were incubated in primary antibodies (and in a cocktail of primary antibodies in case of double fluorescence) prepared in TBS containing 10% normal goat serum/0.3% Triton X-100 at 4°C for 72 h. Sections were then washed in TBS and incubated with relevant secondary antibodies in phosphate buffer (PB) for 2 h and counterstained (3 min) with 0.3% Sudan Black B (dissolved in 70% ethyl alcohol).The sections were then mounted with a preparation of an anti-fade fluorescent medium (DAKO, Carpentaria, CA, USA) and DAPI (4',6-Diamindino-2-Phenylindole, Hydrochloride). Sections were then coverslipped and kept at 4°C until examined.

2.5 Microscopy

Slides were viewed under 20x and 40x objective lenses on a Zeiss AX10 microscope with fluorescence lamps (Carl Zeiss, Inc, GMBH, Jena, Germany). Digital images of single or triple labeling were created using Zeiss image analysis software.

2.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from testicular tissue using the TRIzol reagent (Invitrogen Life Sciences, Carlsbad, CA, USA) and reverse transcribed with a First Strand cDNA Synthesis kit (Fermentas, Osaka, Japan). The primer sequences were synthesised by Fermentas ThermoFisher Scientific, Osaka, Japan, and were as follows: *Kiss1r:* forward 5'-CTCGCTGGTCATCTACGTCA-3', reverse 5'-CGAACTTGCACATGAAATCG-3'; *Kiss1:* forward 5'-CTGGAATCCCTGGACCTCTC-3', reverse 5'-TTGTAGTTCGGCAGGTCCTT-3'.

Amplification of cDNA was carried out in a reaction mixture of 50 µl. The mixture consisted of 3 µl of cDNA, 0.7 µl Taq polymerase, 30 µl RNAse free water, 2.5 µl of each forward and reverse primer, 5 µl reaction buffer and 1.3 µl dNTP. PCR conditions were: 10 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 60°C, 30 s at 72°C using a thermocycler (Whatman Biometra, GmBH, Goettingen, Germany). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as an internal control.

3. Results and Discussion

The present work provides evidence for the expression of Kiss1 and its receptor in adult rhesus monkey testes. It clearly demonstrates the immunoreactive localization of Kiss1 and Kiss1r in the adult rhesus monkey testis. Both immunoreactivities were observed in the specific types of cells within seminiferous tubules (Figures 1A, 3A) (Figures 1A, 2A, 3A, 4A). The immunocytochemical analysis showed localization of both Kiss1 and Kiss1r in germ cells. Kiss1 expression was evident in round spermatocytes and spermatids (Figure 3A). Kiss1 localization within the spermatids was verified with the T4 marker antibody (Figure 4D). Kiss1 and Kiss1r expression were notably absent in Leydig cells. Kiss1r protein was localised to spermatocytes and Sertoli cells (Figure 1A). Co-localization of Kiss1r with inhibin confirmed the Kiss1r in the Sertoli cell (Figure 2D). Our immunocytochemical data were confirmed in our RT-PCR studies where a clear detectable mRNA expression of both *Kiss1* and *Kiss1r* was observed in the testicular tissue and no signal was found for the cDNA negative controls (Figure 5). Clear bands of the expected size (250 bp for *Kiss1* and 171 bp for *Kiss1r)* were observed.

The findings of the present study are in line with observations of an earlier study where Kiss1 and Kiss1r expression was demonstrated in the human testis [6]. These data suggest that kiss1 may act as a paracrine/autocrine factor, during primate spermatogenesis. Accordingly, Kiss1 that is produced by germ cells (spermatocytes) could act in an autocrine manner and/or also modulate Sertoli cells activity; communication between germ-germ cells is also possible. Thus, Kiss1 produced in spermatocytes and spermatids could act on Sertoli cells.

Processes of spermatogenesis in the seminiferous tubules have been found to be under the regulation of both Sertoli cells and germ cells, both by physical interaction and by secretion of biological factors. It is well established that Sertoli cells act to modulate spermatogenesis through various factors such as IGF-I, IGF-2, TGF-α, TGF β, basic FGF, interleukins [20]. Sertoli cells also secrete some glycoproteins such as androgen-binding protein (ABP), transferrin, ceruloplasmin, inhibin, glial cell line-derived neurotrophic factor (GDNF), stem cell factor (SCF) and its ligand c-kit. Vasoactive peptides and tachykinins have also been localized in Sertoli cells [21]. In turn, the capacity of the germ cells to modulate

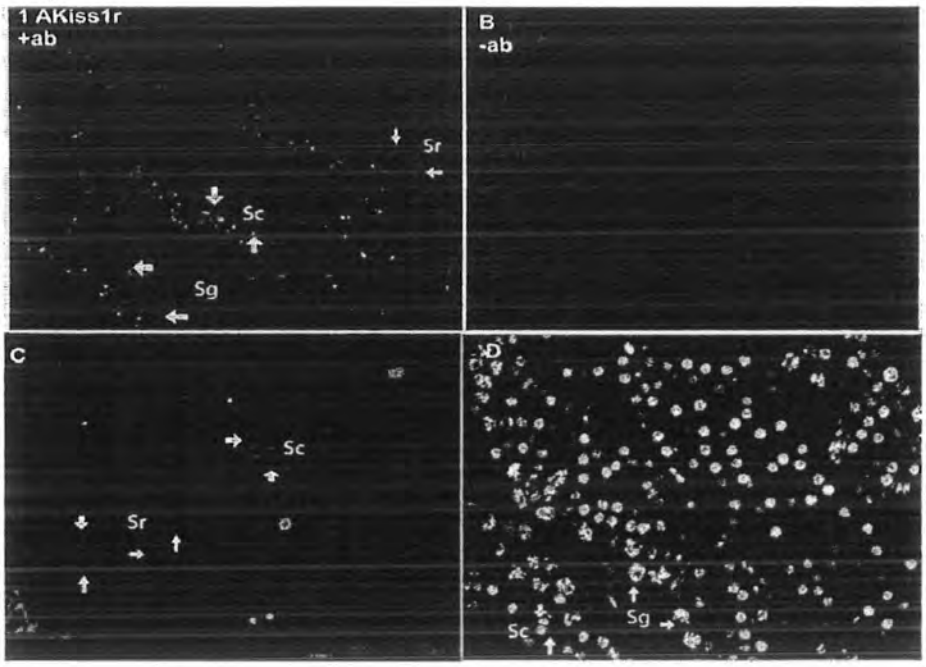

Figure 1. Expression of Kiss1r in seminiferous tubules of the adult rhesus monkey. Kiss1r immunoreactivity was observed in spermatogonia (Sg), spermatocytes (Sc) and Sertoli cells (Sr) (A, 20x). Antibody omitted control (B, 20x) showed no expression. Sertoli cell immunreactivity is shown in a separate section (C, 40x), DAPI was used for cellular nuclei staining (D, 40x).

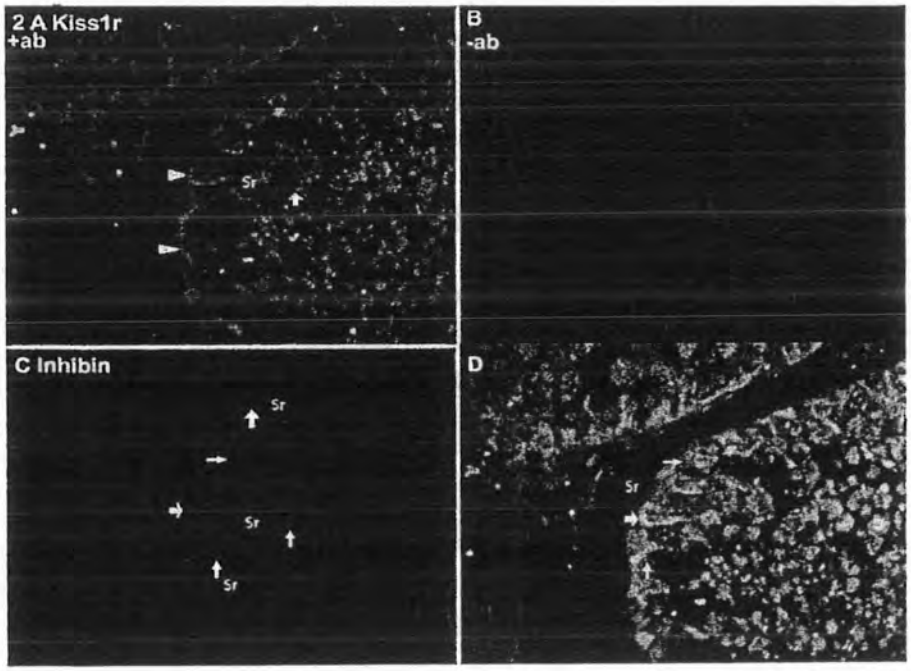

Figure 2. Expression of Kiss1r (A, 20x) and Sertoli cell marker, inhibin (C, 20x) in seminiferous tubules of the adult rhesus monkey. Both Kiss1r and inhibin were localized in Sertoli cells. No immunoreactivity was found in antibody omitted controls (B, 20x). Dual-label cells stand out in yellow when images were merged (D, 20x).

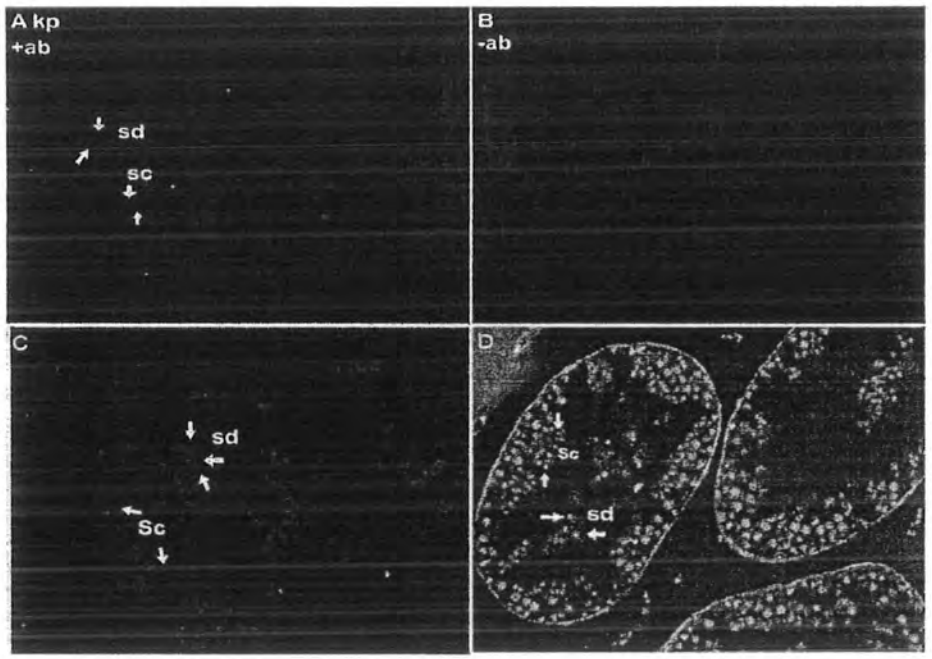

Figure 3. Expression of kisspeptin (kp) in seminiferous tubules of the adult rhesus monkey. Kisspeptin (kp) immunoreactivity was observed in spermatocytes (Sc) and spermatides (Sd) (A, 20x). Antibody omitted control showed no expression (B, 20x). A 40x maginification of a tubule is shown in (C). DAPI was used for cellular nuclei staining (D, 20x).

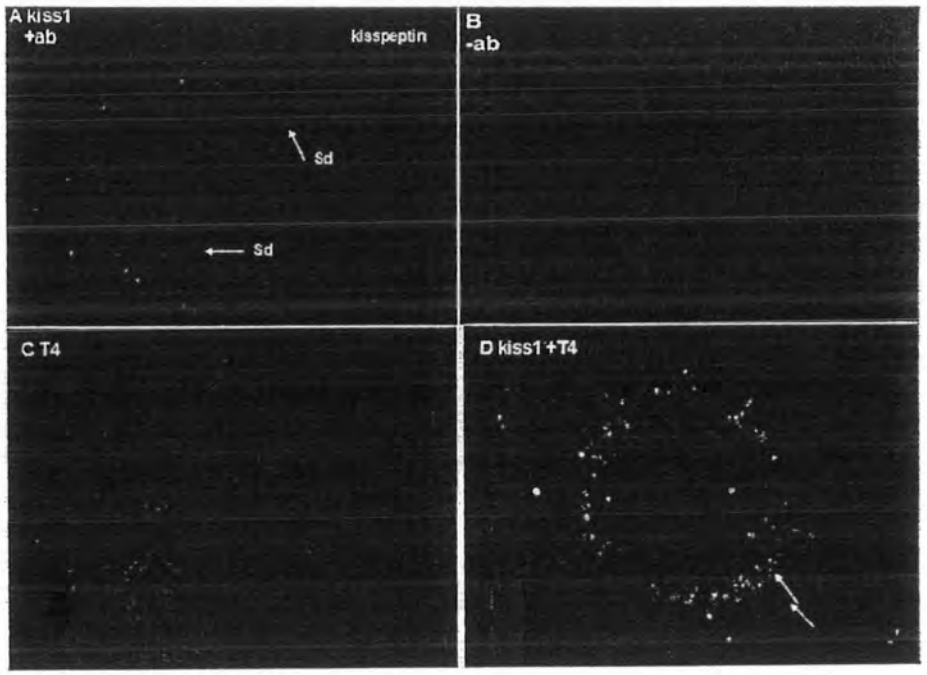

Figure 4. Expression of kisspeptin (kp) (A, 20x) and germ cell marker T4 (C, 20x) in the adult rhesus monkey testis. Both kisspeptin and T4 were localized in spermatocytes (sc) and spermatids (Sd). No immunoreactivity was found in antibody omitted controls (B). Dual-label cells stand out in yellow when images were merged (D, 20x).

Sertoli cell activity has also been postulated [22]. Androgen binding protein, plasminogen activator, and FSH-dependent cAMP production by Sertoli cells have been shown to be affected by germ cells [23-25]. In this regard, germ cells have been shown to secrete follistatin [26,27] and 4.1G, a novel membrane skeletal protein [28]. Our findings suggest that germ cells can also communicate with Sertoli cells with kisspeptin signalling. While the physiological significance of Kiss1 signalling within germ cells and between germ cells and Sertoli cells remains to be determined, recent observation that kisspeptin and Kiss1r have been localized to mature human spermatozoa would strongly suggest so [18]. It has been shown that changes in sperm motility are induced by Kiss1 which is reduced in the presence of the Kiss1r antagonist. These observation suggests that Kiss1, acting *via* Kiss1r, could activate different signal transduction pathways leading to its modulatory role on sperm movement. In summary, present results based on immunohistochemical and RT-PCR data clearly demonstrate presence of both Kiss1 and its receptor in the seminiferous tubules of the adult monkey testis. Expression of *Kiss1* and *Kiss1r* mRNA was also evident in the testis.

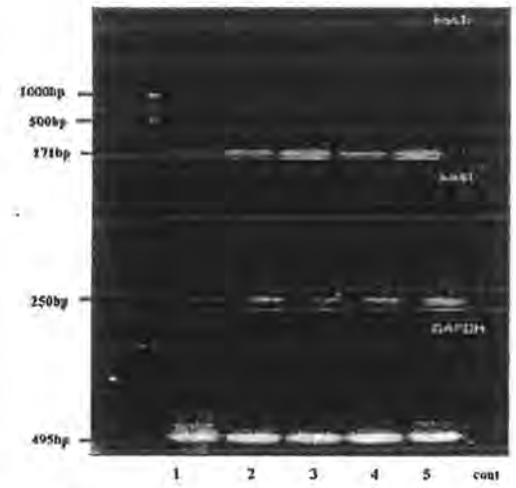

Figure 5. Expression of the mRNAs of kisspeptin and *Kiss1r* in the adult rhesus monkey testis. RT-PCR analysis from five testes samples are presented. Amplicons of 250 bp (kisspeptin) and 171 bp (*Kiss1r*) were obtained. cDNA negative controls of the respective genes showed no bands of corresponding size of positively expressing genes. Amplification of GAPDH served as an internal control.

4. Conclusions

Both Kiss1 and its receptor are expressed in the seminiferous tubules of adult rhesus monkey testis. The expression profile of Kiss1 and its receptor in the testis suggests possible direct involvement in a regulatory network involved to regulate spermatogenesis. The fact that Kiss1 and Kiss1r are restricted to seminiferous tubules suggests a paracrine/autocrine role of Kiss1 signaling. Specific actions of Kiss1 signalling in germ cell-germ cell and germ cell-Sertoli cell interactions and the physiological significance of this remain to be determined.

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References

[1] Tena-Sempere M., KiSS-1 and reproduction: focus on its role in the metabolic regulation of fertility, Neuroendocrinol., 2006, 83, 275-281

[2] Lee D.K., Nguyen T., O'Neill G.P., Cheng R., Liu Y., Howard A.D., et al., Discovery of a receptor related to the galanin receptors, FEBS Letter, 1999, 446, 103–107

[3] West A., Vojta P.J., Welch D.R., Weissman B.E, Chromosome localization and genomic structure of the KiSS-1 metastasis suppressor gene (KISS1), Genomics., 1998, 54, 145–148

[4] Lee J.H., Miele M.E., Hicks D.J., Phillips K.K., Trent J.M., Weissman B.E., et al., KiSS-1, a novel human malignant melanoma metastasis-suppressor gene, J Natl Cancer Inst., 1996, 88, 1731–1737.

[5] Ohtaki T., Shintani Y., Honda S., Matsumoto H., Hori A., Kanehashi K., et al., Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor, Nature, 2001, 411, 613–617

[6] Kotani M., Detheux M., Vandenbogaerde A., Communi D., Vanderwinden J.M., Le Poul E., et al.,

The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G proteincoupled receptor GPR54, J Biol Chem., 2001, 276, 34631–34636

[7] Muir A.I., Chamberlain L., Elshourbagy N.A., Michalovich D., Moore D.J., Calamari A., et al., AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1, J Biol Chem., 2001, 276, 28969–28975

[8] Gottsch M.L., Cunningham M.J., Smith J.T., Popa S.M., Acohido B.V., Crowley W.F., et al., A role for kisspeptins in the regulation of gonadotropin secretion in the mouse, Endocrinology., 2004, 145, 4073–4077

[9] Clarkson J., Herbison A.E., Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons, Endocrinology., 2006, 147, 5817–5825

[10] De Roux N., Genin E., Carel J.C., Matsuda F., Chaussain J.L., Milgrom E., Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54? PNAS USA, 2003, 10972–10976

[11] Seminara S.B., Messager S., Chatzidaki E.E., Thresher R.R., Acierno J.R., Shagoury J.K., et al., The GPR54 gene as a regulator of puberty, N. Engl. J. Med., 2003, 349, 1614–1627

[12] Oakley A.E., Clifton D.K., Steiner R.A., Kisspeptin signaling in the Brain, Endocr Rev., 2009, 30, 713–743

[13] Messager S., Chatzidaki E.E., Ma D., Hendrick A.G., Zahn D., Dixon J., et al., Kisspeptin directly stimulates gonadotropin releasing hormone release via G protein coupled receptor 54, PNAS USA, 2005, 102, 1761–1766

[14] Smith J.T., Li Q., Yap K.S., Shahab M., Roseweir A.K., Millar R.P., et al., Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence, Endocrinology, 2011, 152, 1001-1012

[15] Han S.K., Gottsch M.L., Lee K.J., Popa S.M., Smith J.T., Jakawich S.K., et al., Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty, J Neurosci., 2005, 25, 11349–11356

[16] Irwig M.S., Fraley G.S., Smith J.T., Acohido B.V., Popa S.M., Cunningham M.J., et al., Kisspeptin activation of gonadotropin releasing neurons and regulation of KiSS1mRNA in the male rat, Neuroendocrinol., 2004, 80, 264–272

[17] Castellano J.M., Gaytan M., Roa J., Vigo E., Navarro V.M., Bellido C., et al., Expression of KiSS-1 in rat ovary: Putative local regulator of ovulation, Endocrinology, 2006, 147, 4852–4862

[18] Pinto F., Cejudo-Román A., Ravina C., Fernández-Sánchez M., Martín-Lozano D., Illanes M., et al., Characterization of the kisspeptin system in human spermatozoa, Intel. J. Androl., 2011, 35, 63-73

[19] Ramaswamy S., Seminara S.B., Pohl C.R., DiPietro M.J., Crowley J.W., Plant T.M., et al., Effect of continuous intravenous administration of human metastin 45–54 on the neuroendocrine activity of the hypothalamic-pituitary-testicular axis in the adult male rhesus monkey (Macaca mulatta), Endocrinology, 2007, 148, 3364-3370

[20] Skinner K.M., Norton N.J., Mullaney P.B., Rosselli M., Whaley D.P., Anthony T.C., Cell-cell interactions and the regulation of testis function, Ann N Y Acad Sci., 1991, 637, 354-363

[21] Sofikitis N., Pappas E., Kawatani A., Baltogiannis D., Loutradis D., Kanakas N., et al., Efforts to create an artificial testis: culture systems of male germ cells under biochemical conditions resembling the seminiferous tubular biochemical environment, Hum Reprod Update, 2005, 11, 229–259

[22] Huleihel M., Lunenfeld E., Regulation of spermatogenesis by paracrine/autocrine testicular factors, Asian . J. Androl., 2004, 6, 259-268

[23] Skinner K.M., Growth Factors in Gonadal Development, J. Animal. Sci., 1992, 70, 30-41

[24] Skinner K.M., Nilsson E.E., Bhandari K.R., Cell – Cell signaling in the testis and ovary, In: Edward A.D., Ralph A.B., (Eds.), Handbook of cell signaling, 2nd ed., Academic Press, 2009

[25] Huleihel M., Lunenfeld E., Involvement of intratesticular IL-1 system in the regulation of Sertoli cell functions, Mol. Cell. Endocrinol., 2002, 187, 125–132

[26] Ogawa K., Hasshimoto O., Kurohmaru M., Mizutani T., Sugino H., Hayashi Y., Folllistatin like immunoreactivitiy in the cytoplasm and nucleus of spermatogenic cells in the rats, Euro. J. Endocrinol., 1997, 137, 523-529

[27] Meinhardt A., O'Bryan M.K., McFarlane J.R., Loveland K.L., Mallidis C., Foulds L.M., et al., Localization of follistatin in the rat testis, J. Reprod. Fertil., 1998, 112, 233-241

[28] Terada N., Ohno N., Saitoh S., Saitoh Y., Komada M., Kubota H., et al., Involvement of a membrane skeletal protein, 4.1G, for Sertoli/germ cell interaction, Reproduction, 2010, 139, 883–892

By the end of the Proterozoic Eon eukaryotes, cells that have a nucleus, appeared and were the ancestors of all modern living things. **One of the first extinctions** occurred in the Proterozoic Eon, when oxygen accumulated in the atmosphere and caused those bacteria that required the atmospheric conditions of the Archean Eon to die. The Proterozoic Eon also experienced **two ice ages**, when polar ice became at least a kilometer deep and covered a area extending to the equator.

The Phanerozoic Eon

The Phanerozoic Eon began **540 Million Years Ago and continues into the Present.** The Rocks deposited during the Phanerozoic Eon contain evidence of fossilized hard body.

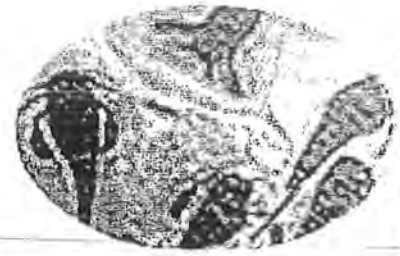
Eras

The Eras are the next largest interval units into which the Geologic Time is divided and represented on the chart. Eras encompass major intervals of Time and are defined based on the fossil life-forms found in the rock layers, and the Law of Superposition.

The Hadean, Archean, and Proterozoic Eons do not have recognized Eras.

There are three Geologic Eras currently identified. The Paleozoic Era, the Mesozoic Era, and the Cenozoic Era.

Each of the names of the Eras reflects the relative stage in the development of life. Paleozoic means old life, Mesozoic means middle life, and Cenozoic means new life.



The Paleozoic Era

The Paleozoic Era is the oldest of the three Eras and dates from **540 Million to 248 Million Years Ago.** During the Paleozoic Era multicelled living things acquired hard body parts, bones, vertebral columns, mandibles, and teeth. Common in the Paleozoic Era were **trilobites, crinoids, brachiopods, fish, insects, amphibians, and early reptiles.**

The Mesozoic Era

The Mesozoic Era extended from **248 Million to 65 Million** Years Ago. The Mesozoic Era was important for the fossil remains of the **dinosaurs and other reptiles** that lived. However, the Mesozoic Era landscape was also occupied by insects, **early mammals,** plants such as conifers and ferns, fish, and finally flowering plants and early birds.